ORIGINS AND REGULATION OF PROGENITOR CELLS

IN THE EMBRYONIC AND ADULT PANCREAS

by

Daniel Kopinke

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STATEMENT OF DISSERTATION APPROVAL

The dissertation of **Daniel Kopinke**

has been approved by the following supervisory committee members:

Charles Murtaugh	, Chair	**5/20/2011** Date Approved
David Grunwald	, Member	**5/20/2011** Date Approved
Richard Dorsky	, Member	**5/20/2011** Date Approved
Suzanne Mansour	, Member	**5/20/2011** Date Approved
Mario Capecchi	, Member	**5/20/2011** Date Approved

and by **Lynn Jorde** , Chair of

the Department of **Human Genetics**

and by Charles A. Wight, Dean of The Graduate School.

ABSTRACT

Type I diabetes is caused by selective loss of insulin-producing β-cells. Identifying and activating an endogenous source of new β-cells could be used to replenish those lost in this disease. The nature and existence of an adult pancreatic stem/progenitor cell population, however, is still controversial. Circumstantial evidence indicates that islet cells arise from embryonic ductal cells. In contrast, the majority of adult islet cells appear to regenerate via self-renewal during postnatal expansion and adult homeostasis. That ducts could also give rise to new beta-cells in the adult (neogenesis) was recently suggested in the context of pancreatic ductal ligation (PDL) injury. The Notch signaling pathway inhibits islet development and promotes progenitor cell maintenance during early pancreatic organogenesis, acting primarily through its target gene *Hes1*. While *Hes1* is broadly expressed in the embryonic pancreas, only rare Hes1-expressing cells can be found in the adult organ, among mature ducts and centroacinar cells (CACs), the latter of which have been proposed to represent adult progenitors. This thesis aims to test directly the ability of duct cells to generate islet cells, and to determine the biological function of $Hes1^{+}$ duct cells. For this, we performed lineage tracing and genetic manipulation using two novel Cre-lines, $Muc1^{IC2}$, which marks exocrine cells, and $Hes1^{C2}$, which marks $Hes1^{+}$ cells. Our work has uncovered three major phases in the development of pancreatic islet cells: (i) initially, new islet cells originate from Notch-responsive exocrine cells, (ii) later in embryogenesis, Notch signaling needs to be down-

regulated in exocrine cells for islet neogenesis to proceed and (iii) from birth onwards, islet cells are maintained by replication of pre-existing cells with no detectable influx from neogenesis. Additionally, our findings demonstrated that early *Hes1*$^{+}$ cells represent multipotent progenitors and that their immature state is maintained through active Notch signaling. Interestingly, later in development Notch promotes duct specification of *Hes1*-expressing bipotent exocrine progenitors. In the mature pancreas, *Hes1* expression persists in Notch-sensitive centroacinar cells, which act as facultative exocrine-specific progenitor cells. Thus, the research described in this thesis determines the identity of embryonic and adult pancreatic progenitor cells, and demonstrates that these cells utilize Notch signaling for maintenance of their undifferentiated state.

This thesis is dedicated to my parents, Mechthild and Hans-Joachim Kopinke.

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CHAPTER 1

INTRODUCTION

Type 1 diabetes results from the specific loss of insulin-producing beta (β)-cells, and therefore represents a candidate disease for cell replacement therapy. Transplantation of islets can serve as a potential cure for this disease. The relatively low abundance of endogenous β-cells within the pancreas and the shortage of donor organs, however, makes finding new ways of generating functional β-cells a necessity. One intriguing possibility to treat this disease is by generating new β-cells from an endogenous source. Whether stem or progenitor cells exist in the adult pancreas, either dedicated to islet differentiation or recruited by injury, remains controversial. Histological evidence suggests that β-cells and other islet cells originate from multipotent progenitor cells within the embryonic ducts (a process termed neogenesis). In contrast, genetic lineage tracing studies indicate that β-cells are maintained in the mature pancreas by self-renewal. Recent evidence, however, suggest that new β-cells could also arise from duct cells following pancreatic injury. Whether these cells represent terminally differentiated ducts or progenitors residing within the ductal network is still unknown.

The first half of this thesis focuses on the lineage relationship between ducts and islet cells during embryogenesis and adulthood, emphasizing two main questions: (1) What is the origin of the new β-cells that arise in large numbers shortly after birth? (2) Is

there any contribution to new islets from the exocrine tissue (duct and acinar cells) during adult homeostasis or regeneration?

The second half of this thesis addresses the question whether the pancreas contains an adult stem/progenitor cell. Duct cells have been proposed to be the origin of new β-cells after injury, and centroacinar cells to represent adult progenitor cells. The latter cell type has been shown to possess multilineage differentiation potential in vitro, but confirmation in vivo is still lacking. The Notch signaling pathway is involved in maintenance of stem cells in several organs. In the pancreas, Notch plays an important role in cell specification and differentiation in that it represses islet development and promotes progenitor cell maintenance during early pancreas organogenesis, acting primarily through its target gene *Hes1*. *Hes1* is broadly expressed in undifferentiated cells of the early pancreas, while in the adult it is expressed by scattered ductal and centroacinar cells. This thesis seeks to elucidate (3) whether *Hes1*$^+$ cells are multipotent throughout pancreatic organogenesis and (4) whether the rare *Hes1*$^+$ cells in the mature pancreas represent adult progenitors similar to their embryonic counterparts.

By examining the extent to which ducts can contribute to new β-cells, we shed light on the question whether human ductal cells could serve as a source for beta-cell replacement therapy in type I diabetes. Moreover, by studying the Notch pathway in closer detail, we now better understand its functions not only in development, but also in adult pancreatic disease. This thesis also addresses fundamental biological questions about the relationship between embryonic development and adult homeostasis, applicable to other organ systems such as the liver and intestines.

CHAPTER 2

PANCREATIC STEM CELLS

Reprint of: Murtaugh, L. C., and Kopinke, D. (2008). Pancreatic stem cells. StemBook. Reprinted with permission from StemBook.

Pancreatic stem cells*

L. Charles Murtaugh and Daniel Kopinke§, Department of Human Genetics, University of Utah, Salt Lake City, UT 84112, USA

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Abstract

Controversy over whether pancreatic islet cells arise from adult stem or progenitor-like cells actually predates the discovery of insulin, and the recent use of islet transplantation to treat diabetes has only intensified interest in this question. Recent breakthroughs, particularly those based on Cre-loxP lineage-tracing in the mouse, have resolved some aspects of this controversy, but not all. We now know that insulin-producing β-cells and other islet cells derive from multipotent progenitors in the embryo, but that their maintenance and expansion in postnatal life is driven primarily by proliferation of existing differentiated cells. This appears to be true even during regeneration, and seems to apply to the exocrine acinar cells as well as islets. Following pancreatic duct ligation, however, islet precursors re-appear in the injured pancreas, arising from ducts and differentiating into new islet cells. Thus, while the pancreas does not normally rely on classical stem cells, a stem cell-like mechanism for new islet differentiation may be inducible under specific circumstances. Understanding the signals that promote β-cell formation in the embryo and adult should facilitate efforts to derive clinically-useful β-cells in vitro, either from adult ducts or embryonic stem cells.

1. Introduction

Type I diabetes is caused by the autoimmune destruction of pancreatic β-cells, and has emerged as a case study for stem cell-based "regenerative medicine." Its selling point is the idea that the location of β-cells within the pancreas is irrelevant to their ability to regulate blood sugar through insulin release. Moved elsewhere, so long as they have

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§Corresponding author (email: murtaugh@genetics.utah.edu)

access to the circulation, β-cells should function to maintain glucose homeostasis – a hypothesis amply supported by animal studies (Ballinger and Lacy, 1972), and now the basis for clinical islet transplantation in humans (Naftanel and Harlan, 2004).

Islet transplantation confronts formidable hurdles as a treatment for type I diabetes, such as blocking autoimmunity and preserving graft function. When these difficulties are overcome, however, the approach will still be hampered by the scarcity of cadaver-derived islets. Three potential solutions have been proposed: first, to enhance replication of islet cells in vitro, "stretching" the limited supply; second, to isolate adult stem cells from the pancreas that can expand and produce new β-cells; third, to manipulate embryonic stem (ES) cells so that they adopt a β-cell identity.

The first of these approaches, recently discussed elsewhere (Dhawan et al., 2007; Nir and Dor, 2005), is beyond the scope of this review, although we will discuss the contribution of β-cell proliferation to islet regeneration following injury. With respect to the second approach, we will consider the existence of stem cells in the adult pancreas, and discuss how they might be exploited clinically. Finally, we will discuss recent advances toward β-cell derivation from ES cells, and consider how this approach might benefit both basic and clinical researchers. As any of these strategies will depend on understanding normal pancreas development and homeostasis, it is here that we will begin our review.

2. The normal pancreas: embryonic development and adult homeostasis

The mammalian pancreas arises from two evaginations in the posterior foregut, one dorsal and one ventral, which expand into the tail and head, respectively, of the mature organ. The first evidence of pancreas development, at embryonic day 8.5 (E8.5) in the mouse, is expression of the homeodomain transcription factor *Pdx1* within the cells that will evaginate to form the pancreatic buds (Ohlsson et al., 1993). Shortly thereafter, these undifferentiated progenitor cells begin to express the bHLH transcription factor *Ptf1a* (Burlison et al., 2008; Kawaguchi et al., 2002), as well as the digestive enzyme *Cpa1* (Zhou et al., 2007). Genetic lineage-tracing, using the Cre-loxP system, indicates that essentially all mature pancreatic cells, including acini, ducts and islets, arise from these $Pdx1^{+}$/$Ptf1a^{+}$ progenitor cells (Gu et al., 2002; Kawaguchi et al., 2002). Moreover, in both mice and humans, pancreas growth and differentiation are nearly abolished when either of these genes is mutated (Jonsson et al., 1994; Kawaguchi et al., 2002; Krapp et al., 1998; Sellick et al., 2004; Stoffers et al., 1997). Although there have been few thorough studies of human embryonic and fetal pancreas development, the weight of evidence indicates overall similarity to that of rodents (Castaing et al., 2005; Piper et al., 2004; Sarkar et al., 2008).

Space does not permit a full discussion of the complexities of pancreas development, which have been reviewed elsewhere (Collombat et al., 2006; Dhawan et al., 2007; Murtaugh, 2007). We will focus instead on the issue of pancreatic lineages, as it relates directly to the question of pancreatic stem cells. Figure 1 summarizes a number of mouse lineage-tracing experiments, the important elements of which are as follows:

- Nearly all mature pancreatic cells arise from primitive cells expressing *Pdx1* and *Ptf1a* (Gu et al., 2002; Kawaguchi et al., 2002).
- At least some of these cells, expressing low levels of the acinar enzyme *Cpa1*, are self-renewing, multipotent progenitors (Zhou et al., 2007).
- Transient expression of the bHLH transcription factor *Neurog3*/*Ngn3* marks specification of these progenitors to the islet lineage (Gu et al., 2002).
- At E13.5, coincident with massive differentiation of both endocrine and exocrine cells (the so-called "secondary transition"; Pictet and Rutter, 1972), *Cpa1* expression becomes acinar-specific, and no longer marks multipotent progenitors (Zhou et al., 2007).
- In the postnatal pancreas, absent injury, there is no evidence for stem or progenitor cell contribution to either endocrine or exocrine cells, the growth of which can be accounted for by division of existing differentiated cells (Desai et al., 2007; Dor et al., 2004; Strobel et al., 2007).

These last findings represent only the latest word in a debate about adult "neogenesis," i.e. the differentiation of new islets from stem or precursor cells, that has been active since the late 19th-century (Bensley, 1911; Bonner-Weir and Weir, 2005). Fueling this argument are two long-standing observations: first, that islet (and β-cell) mass continues to increase after birth, and second, that islet cells and their precursors in the embryo are found to reside in or near ductal structures (see Figure 2). If embryonic ducts can differentiate into islets, why not those of the adult? On the other hand, the rates of postnatal islet proliferation and death are generally low, and there is no obvious proliferative

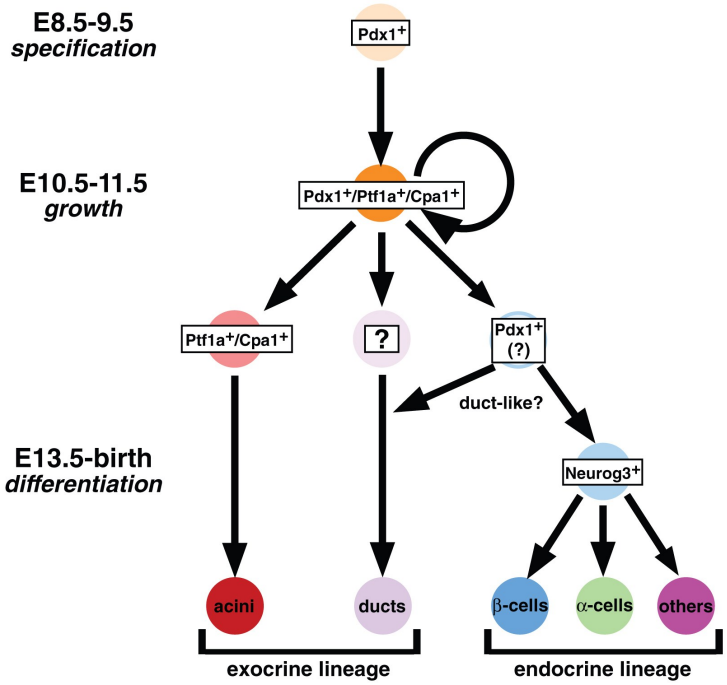

Figure 1. Pancreatic lineages in the mouse. Pancreas specification within the foregut endoderm is first indicated by expression of the transcription factor *Pdx1*, which marks the progenitors of all exocrine and endocrine cell types. As the embryonic organ grows, multipotent progenitor cells also express the transcription factor *Ptf1a* and the digestive enzyme *Cpa1*. These progenitors are later segregated into specific sub-lineages, prior to differentiation. Acinar cells arise from precursors that express high levels of *Ptf1a* and *Cpa1*, while islet cells arise from precursors that transiently express the transcription factor *Neurog3*. Although *Neurog3*-expressing cells arise from duct-like epithelial progenitors (see Figure 2), the lineage relationship between these structures and the ducts of the mature organ is unclear (question marks).

niche such as is found in the skin or intestines. As the differentiated cells of those tissues are short-lived, they must be replenished by a classical stem cell compartment; the slow turnover of islet cells may not require such a mechanism. On the other hand, as we will discuss, β-cells exhibit some capacity for regeneration after injury, and it is possible that some of this capacity reflects the activity of "facultative stem cells" similar to those proposed to exist in the liver (Michalopoulos, 2007).

The field is therefore divided into camps that we term "expansionist," believing that β-cell mass increases via replication of existing β-cells, and "neogenicist," arguing that at least some of this increase reflects differentiation of islet precursor cells. Two lines of evidence support the expansionist position. The first concerns *Neurog3*, the expression of which marks the precursors of all islet cells (Gu et al., 2002), and which is necessary and sufficient for endocrine specification in vivo (Apelqvist et al., 1999; Gradwohl et al., 2000; Johansson et al., 2007; Schwitzgebel et al., 2000). *Neurog3* expression is thus the sine qua non of islet development, yet it is expression is undetectable in the adult pancreas, including ducts (Gradwohl et al., 2000; Lee et al., 2006; Schwitzgebel et al., 2000). These findings, or more precisely the lack thereof, suggest that adult islet precursor cells do not exist.

Neogenicists might argue that the slow postnatal increase in β-cell mass requires very few islet precursors at any one time, perhaps easy to overlook. Furthermore, as we discuss below, it may be that neogenesis "ramps up" only when β-cell mass is lost to injury or disease. The strongest expansionist counter-argument is the seminal

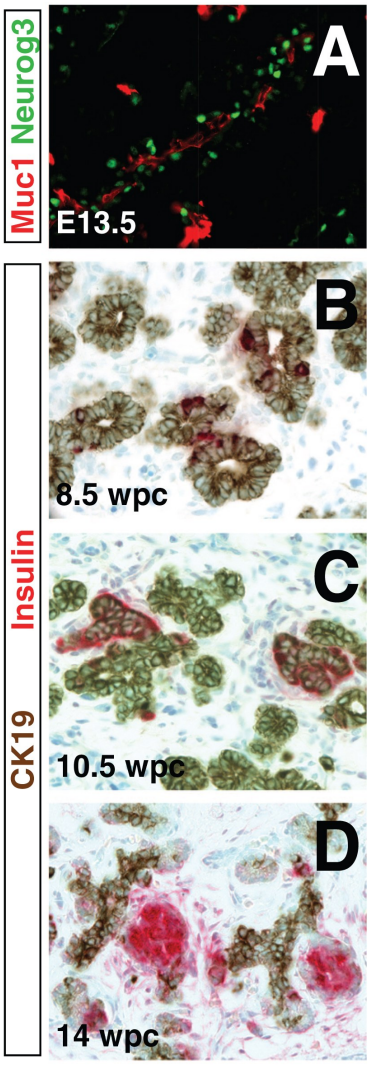

Figure 2. Relationship between developing islet and duct cells. Co-immunofluorescence staining of Neurog3 (green) and the duct marker Muc1 (red), in E13.5 mouse pancreas, reveals the intimate relationship between islet precursor cells and duct-like structures A. This can also be appreciated from co-immunohistochemistry for insulin (red) and the duct marker cytokeratin-19 (brown) on sections of human fetal pancreas B–D. At early gestational ages (8.5–10.5 weeks post-coitum), β-cells are located within ductal structures, and co-express insulin and CK19. At later ages (14 wpc-adult), CK19-negative β-cells aggregate outside the ductal network. Panels B–D are adapted from Piper et al. (Piper et al., 2004). B–D copyright Society for Endocrinology (2004), reproduced by permission.

lineage-tracing study of Dor and colleagues (Dor et al., 2004), the results of which are schematized in Figure 3A.

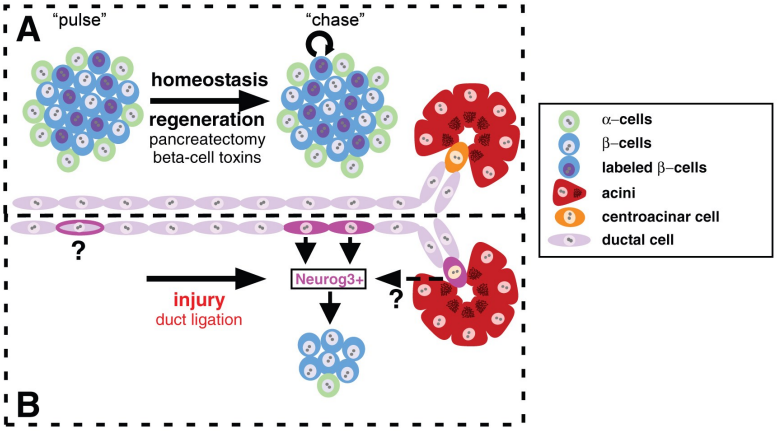

Figure 3. Sources of new adult β-cells. A. "Pulse-chase" lineage-tracing studies (Dor et al., 2004; Nir et al., 2007) involve Cre-loxP-induced marking of pre-existing β-cells with heritable expression of a neutral transgene such as alkaline phosphatase (purple nuclei within blue β-cells, left). During homeostasis and growth, or following removal of β-cells by partial pancreatectomy or ablation with diphtheria toxin, β-cell numbers are maintained or restored by division of pre-existing β-cells (similar fraction of labeled β-cells after growth/injury, right). B. While there is no evidence for neogenesis from ductal cells in the uninjured pancreas (question mark, left), new Neurog3-expressing islet precursors appear after duct ligation, giving rise to new islet cells including β-cells (right) (Xu et al., 2008). Although Neurog3^{+} cells seem to derive from ducts, formal lineage-tracing has not ruled out the possibility that they might arise from centroacinar or acinar cells (question mark, right). This study predicts that pulse-chase labeling of pre-existing β-cells, before duct ligation, should reveal no contribution to the duct-derived β-cells that arise from injury-induced neogenesis (unlabeled islet cell nuclei).

Briefly, these researchers used an inducible Cre-loxP system to heritably mark a fraction of islet cells (the "pulse," indicated as purple nuclei on the left side of Figure 3A). This marking was performed while the mice were young (6-8 weeks), and a subset of the mice were immediately sacrificed and analyzed for labeling index. The remaining mice were allowed to age for up to a year (the "chase"), during which time β-cell numbers increased several fold. If this increase was due to neogenesis, the β-cells labeling index should have fallen due to influx of unmarked islet precursor cells. Instead, the labeling index remained constant during the chase (see Figure 3A, right), supporting the expansionist model (Dor et al., 2004).

This model has since acquired independent support from alternative pulse-chase paradigms, using novel lineage-tracing techniques (Brennand et al., 2007) or sensitive measurement of proliferation kinetics (Teta et al., 2007). These studies present a powerful contrast to those based mainly on histology. For example, small β-cell clusters, or solitary β-cells adjacent to ducts, are frequently interpreted as products of neogenesis (Bouwens and Pipeleers, 1998; Wang et al., 1993). Analysis of these structures by lineage-tracing (Dor et al., 2004) and cell proliferation kinetics (Teta et al., 2007), however, strongly suggests that the majority of them originate from pre-existing β-cells.

Even if neogenesis does not make a major contribution for normal β-cell maintenance and growth, the process may be activated following damage to the pancreas or to the β-cells themselves. We will examine this possibility in the next sections, but we should first note that there is no evidence for such compensatory behavior by progenitor cells of the embryonic pancreas. Instead, if a subset of progenitors is destroyed in utero, by expression of a toxic transgene, the organ remains proportionally smaller in mass (Stanger et al., 2007). If compensation for lost cells does occur in adults, the underlying phenomenon is unlikely to simply recapitulate embryonic organogenesis.

3. Pancreatic regeneration: re-growth or re-differentiation?

The pancreas is no liver – if half is excised, the remnant will not double in size. The same applies to β-cells, which can be targeted for experimental destruction by various means. Nonetheless, both the pancreas and its β-cells

are capable of at least modest regeneration following surgical, chemical or transgenic injury, in some cases sufficient to reverse hyperglycemia (reviewed in Bouwens and Rooman, 2005). Do these situations, in which the normally slow mitotic engine of the adult organ shifts into high gear, reveal a role for pancreatic stem cells?

For several decades, researchers have generated rodent models of diabetes by poisoning β-cells with relatively selective compounds, most commonly streptozotocin (Bouwens and Rooman, 2005; Lenzen, 2008). As a rule, the effects of these treatments are irreversible, although development of diabetes is more gradual with lower doses and younger animals (Junod et al., 1969; Like and Rossini, 1976). Nonetheless, β-cell numbers do appear to rebound moderately following high-dose streptozotocin (STZ) treatment of young rats, or low-dose treatment of adult mice (Bonner-Weir et al., 1981; Dutrillaux et al., 1982; Krishnamurthy et al., 2006; Teta et al., 2005). This system exemplifies a problem in the field: β-cell regeneration occurs only after less-than-complete destruction of existing cells. It is therefore hard to know if new β-cells arise via neogenesis or expansion of residual survivors. While analysis of proliferation kinetics supports the latter model (Teta et al., 2005; Wang et al., 1994), there have not yet been any genetic marking experiments in which pre-existing β-cells are marked and traced through the regeneration process.

The fact that normal animals almost never recover from STZ-induced diabetes makes it a questionable model for regeneration. As an alternative, Nir et al (Nir et al., 2007) developed a bi-transgenic mouse strategy in which the diphtheria toxin A (DTA) subunit is expressed, in a regulateable fashion, within mature β-cells. The potent and cell-autonomous lethality of DTA produces an all-or-nothing model of β-cell killing, in which individual cells either express DTA and die, or fail to express the gene and survive. In this system, transgene activation causes rapid development of diabetes, which is reversible upon transgene deactivation. The return to normoglycemia takes several weeks, and is accompanied by an almost complete restoration of β-cell mass. Importantly, simultaneous genetic lineage-tracing reveals that the new β-cells arise via mitotic expansion of pre-existing β-cells (Nir et al., 2007).

What about more extreme models of β-cell and pancreatic damage and regeneration? Partial pancreatectomy has been a favorite model of pancreatic regeneration for many years, and rodents can restore lost β-cell mass following removal of the majority of the organ (Brockenbrough et al., 1988). Several lines of evidence, however, have converged to indicate that this process – which is slow and often incomplete – is driven by expansion rather than neogenesis. First, when existing β-cells are genetically marked prior to pancreatectomy, identical labeling indices are found before and after regeneration (Dor et al., 2004). Second, proliferation kinetic studies indicate enhanced β-cell mitosis post-pancreatectomy, and no sign of differentiation from ductal progenitors (Teta et al., 2007). Finally, *Neurog3* expression is not re-activated during regeneration, as might be expected for a process that recapitulates fetal neogenesis (Lee et al., 2006).

One caveat to these mouse studies is that they have involved 50–70% pancreatectomy, rather than the subtotal (90–95%) removal that has been reported to produce neogenesis in rats, with histological evidence of duct-to-islet differentiation (Bonner-Weir et al., 1993). Whether *Neurog3* is re-expressed in the rat model is not yet known, but a positive result would be especially compelling given recent evidence, discussed below, that *Neurog3*-dependent neogenesis can be induced in adult mouse ducts (Xu et al., 2008). A further caveat, possibly more important, is that the mouse studies might not have had sufficient statistical power to exclude a minor contribution from ductal neogenesis. For this reason, it would be extremely helpful to have an inducible, duct-specific Cre line, analogous to that used by Dor and colleagues (Dor et al., 2004) to mark pre-existing β-cells. By marking duct cells prior to injury, one should be able to detect even a minor contribution on their part to regenerated β-cells. Until such experiments have been performed, it seems prudent to conclude that regeneration of lost β-cells generally involves expansion of those cells left behind, rather than neogenesis (see Figure 3A). β-cell proliferation is known to accelerate under physiological conditions of high insulin demand, such as pregnancy, and this same mechanism may be employed during regeneration (Bouwens and Rooman, 2005).

Before moving on, we should pause to consider the question – less well studied – of whether stem cells exist for the exocrine pancreas. The exocrine pancreas can also regenerate following partial pancreatectomy, although the extent appears to be less than that of the β-cells (Brockenbrough et al., 1988; Desai et al., 2007). Other injury models exist for acinar cells, such as treatment with high levels of the secretagogue caerulein, in which acinar cells are initially destroyed and later recover (Willemer et al., 1992). Remnant duct and/or acinar cells appear to revert to a more progenitor-like state in these models; for example, *Pdx1* expression is highly upregulated, and their proliferation rate increases (Jensen et al., 2005; Sharma et al., 1999). Lineage-tracing studies indicate that the recovery in acinar mass in these models occurs not via differentiation of new acini from ductal or other progenitors, however,

but by expansion of those acinar cells that survived injury (Desai et al., 2007; Strobel et al., 2007). The acinar cells themselves generally do not change fate during this process, although after repeated caerulein treatment a small number of acinar cells seem to adopt a duct-like phenotype (Desai et al., 2007; Strobel et al., 2007). Proliferation of existing differentiated cells therefore appears to be the rule for growth and regeneration in the adult pancreas, endocrine and exocrine.

4. The exception that proves the rule: pancreatic duct ligation

All doubt regarding duct-to-islet neogenesis could be resolved with a duct-specific inducible Cre transgenic mouse. While no such mouse is yet described, the impetus for its development has become overwhelming in light of recent work providing very strong evidence of in vivo β-cell neogenesis (Xu et al., 2008). This study made use of pancreatic duct ligation (PDL), in which the main duct of the splenic lobe is tied off, causing massive death of distal acinar cells. In rats, PDL induced β-cell mass within the ligated portion to double within a week, while the unligated portion remains unchanged (Wang et al., 1995). This increase is too rapid to be accounted for by the observed proliferation rate of existing β-cells, and the model presents histological evidence of neogenesis from ducts (Wang et al., 1995). Importantly, PDL differs from the injury models described above in that it does not involve loss of pre-existing β-cells, nor even transient hyperglycemia (Wang et al., 1995; Xu et al., 2008). Indeed, Frederick Banting exploited the acinar deletion caused by PDL, and the resulting elimination of contaminating proteases, in his initial efforts to isolate insulin (Banting, 1925). We are therefore reluctant to desribe the PDL model as "regeneration" per se, as it involves production of extra β-cells rather than the replacement of β-cells lost to injury. In addition, the signals involved are probably distinct from those that couple β-cell mass to physiological insulin demand, as there is no hyperglycemia, and the unligated portion of the organ is completely unaffected. Presumably some very local signal is activated after duct ligation, perhaps triggered by acinar cell death or inflammation, and perhaps more similar to the signals that regulate embryonic islet development than those normally involved in maintaining adult β-cell mass.

Neurog3 expression studies provide the best evidence for neogenesis following PDL (Xu et al., 2008). As noted above, *Neurog3* is not normally expressed in the adult pancreas, even after pancreatectomy. Following PDL, however, several assays indicated its re-expression: realtime PCR analysis of total pancreatic RNA, immunostaining with a monoclonal anti-Neurog3 antibody, and expression of three independent *Neurog3*-driven reporter genes (both transgenic and knock-in). As the perdurance of these reporters exceed that of *Neurog3* itself, Xu and colleagues were able to show that their expression initiated in duct cells and persisted into new islet cells, including β-cells. Furthermore, lentiviral knockdown of *Neurog3* in the ligated pancreas blocked the doubling of β-cell mass, consistent with the need for *Neurog3* in fetal islet development (Gradwohl et al., 2000; Xu et al., 2008).

Together, the results of this study strongly indicate that duct ligation induces β-cell neogenesis. Furthermore, if the β-cell "pulse-chase" paradigm was to be applied to this system (Dor et al., 2004), we would predict a large increase in the proportion of unlabeled β-cells, reflecting a doubling of β-cell mass due primarily to neogenesis (see Figure 3B). This experiment has not yet been performed, although the reagents clearly exist. As noted above, many uncertainties regarding neogenesis could be clarified with a duct-specific inducible Cre driver line, which should robustly confirm the ductal origin of new β-cells formed after PDL. Assuming that the conclusions of Xu and colleagues (Xu et al., 2008) are confirmed by lineage-tracing of duct cells, it raises three immediate questions: first, what signals are responsible for evoking *Neurog3* re-expression and islet neogenesis; second, is there a special subset of duct cells that can undergo islet differentiation, or does the abnormal environment, post-PDL, trigger stochastic conversion of normal duct cells into islet precursors; third, can these signals can be used to induce β-cell differentiation from human duct cells?

Although there is no obvious answer to the first question, it should be noted that islet neogenesis appears to occur in the ducts of transgenic mice expressing interferon-γ in β-cells; as in the PDL model, the pancreata of these mice exhibit inflammation and immune cell infiltration, but not hyperglycemia (Gu et al., 1994; Gu and Sarvetnick, 1993). (Whether *Neurog3* is upregulated in these mice is unknown.) An inflammatory signal may therefore comprise part of the trigger for ductal neogenesis. The second question is equally hard to answer; interestingly, a similar issue has been raised regarding the role of astrocytes as adult neural stem cells: are the neurogenic astrocytes intrinsically unique, or is their stem cell behavior determined by their niche (Riquelme et al., 2008)?

The third question, of whether human duct cells can be converted into β-cells, is the subject to which we now turn.

5. Duct-to-islet differentiation: in vitro veritas?

The possibility of deriving β-cells in culture, via differentiation of ductal or other adult progenitor cells, has been intensely studied over the past several years, with successes reported using both mouse and human tissue (Bonner-Weir et al., 2000; Gao et al., 2005; Ramiya et al., 2000; Seaberg et al., 2004; Suzuki et al., 2004; Zulewski et al., 2001). None of these methods has yet achieved widespread use, however, and two general problems seem to bedevil studies in this area. First, not knowing the signals that trigger endocrine differentiation, in utero or in adults (e.g. following PDL), makes it hard to design rational culture conditions to optimize the process. Second, most pancreatic cell preparations – even those from which intact islets have been separated – are contaminated by pre-existing β-cells. Survival and expansion of those β-cells during culture will make it hard to detect true neogenesis. Indeed, one group has reported that prior removal of rare islet cells from a duct preparation, by sorting against the islet cell surface marker NCAM, almost completely eliminated in vitro neogenesis (Gao et al., 2005). This might imply that a phenomenon widely interpreted as neogenesis actually represents the expansion of pre-existing islet cells in vitro, or else that islet precursors express a marker, NCAM, generally assumed to be specific to differentiated endocrine cells.

Two other recent studies, meanwhile, do provide strong evidence for islet differentiation in culture from adult non-islet cells; although their approaches differ, they converge on one intriguing point. Hao and colleagues (Hao et al., 2006) found that culturing adult human pancreas cells with the aminoglycoside antibiotic G418 would eliminate both mesenchymal cells as well as pre-existing β-cells. The remaining non-endocrine pancreatic epithelial cells (NEPECs) did not form β-cells in culture, or upon transplantation to the mouse kidney capsule. When co-transplanted with human fetal islet cell clusters (a mix of islet, epithelial and mesenchymal tissue), however, 10-20% of NEPECs differentiated into insulin$^+$ β-cells (Hao et al., 2006). In separate work, Yatoh and colleagues (Yatoh et al., 2007) purified duct cells from adult human pancreas by sorting for the duct cell surface antigen CA19-9. The purified duct cells did not form β-cell differentiation in culture, or when transplanted to the mouse kidney capsule; when aggregated with pancreatic stromal fibroblasts prior to transplantation, however, ∼1% of the duct cells underwent β-cell differentiation (Yatoh et al., 2007).

Although these studies used different methods to isolate starting material (and report very different efficiencies of β-cell neogenesis), they share the fact that neogenesis occurred only following transplantation to the kidney capsule, and required co-transplantation with "helper cells." The kidney capsule is known to be a favorable environment for differentiation of transplanted fetal islets (Castaing et al., 2005; Hayek and Beattie, 1997), and the expansion of embryonic islet progenitors is promoted by association with mesenchymal cells (Duvillie et al., 2006; Miralles et al., 2006). Thus, the progenitor-like behavior observed in these studies parallels that seen in utero, including the unfortunate fact that it works best under conditions that are not easily mimicked in vitro.

Together with the duct ligation work described above (Xu et al., 2008), these studies suggest that the short supply of donor islets could be "stretched" by expansion and differentiation of progenitor-like cells within the leftover exocrine tissue. (A shortcoming of the culture studies, however, is that they have not yet documented *Neurog3* expression by the putative precursor cells.) The fact that neogenesis only occurs after transplantation also raises concerns about its eventual clinical applicability; something critical happens in vivo that cannot be reproduced under controlled conditions in vitro. As we discuss in the next and final section, this in vivo/in vitro dichotomy also applies to another proposed source of new β-cells, embryonic stem cells.

6. From embryonic stem cell to beta-cell

This review has focused on what goes on inside the pancreas, and in particular whether one can obtain stem cells – or cells with similarly useful properties – from the pancreas. In this penultimate section, we will take a detour to consider the opposite question: can one obtain pancreas from stem cells, specifically embryonic stem (ES) cells? A full consideration of the ES cell-to-pancreas literature is outside the scope of this review; we refer interested readers to several other, excellent reviews that treat the topic at length, including its numerous pitfalls and false starts (Madsen, 2005; Murry and Keller, 2008; Spence and Wells, 2007).

The attraction of ES cells as a potential source of pancreatic tissue, including islets, is their capacity to differentiate into any cell type. This is proven for mouse ES cells, which can reconstitute an entire embryo (Nagy et al., 1993), and correlative evidence suggests that their human counterparts (hESCs) are similarly pluripotent (Odorico et al., 2001).

The fact that ES cells can become pancreas in a mouse, however, does not mean that they are easily compelled to do so in a dish. Work in a number of "directed differentiation" paradigms, including pancreas, suggests that success depends on recapitulating, in vitro, the steps that occur during normal development in vivo (Murry and Keller, 2008; Spence and Wells, 2007). Among the first steps of pancreas development is the induction of definitive endoderm, which requires signaling by transforming growth factor-β (TGFβ) proteins of the activin or Nodal families (Gamer and Wright, 1995; Henry et al., 1996; Tremblay et al., 2000). Applying this insight to ES cell differentiation, it has recently been shown that both mouse and human ES cells can be robustly converted to endoderm by treatment with activin or Nodal (D'Amour et al., 2005; Kubo et al., 2004; Yasunaga et al., 2005).

Several groups have since exploited additional known regulators of pancreas development, such as FGF10 and retinoic acid, to push ES cells further down the path of β-cell differentiation (D'Amour et al., 2006; Jiang et al., 2007; Micallef et al., 2005; Nakanishi et al., 2007). The work of D'Amour and colleagues (D'Amour et al., 2006) is particularly compelling, as it was performed entirely in monolayer culture, and resulted in highly efficient generation of cells expressing pancreatic progenitor markers. Although insulin-producing cells did develop in these cultures, however, they appeared to be immature, and were not glucose-responsive. More recently, this group and another have transplanted hESC-derived pancreatic progenitors into the mouse kidney capsule or fat pad, and shown that these environments can promote differentiation of mature, functional human β-cells (Kroon et al., 2008; Shim et al., 2007). The nature of the signals provided at the site of transplantation are unknown, although, as noted above, very similar findings were made with cells derived from the adult pancreatic duct (Hao et al., 2006; Yatoh et al., 2007). One potentially important element present in vivo, and absent from in vitro cultures, is the host endothelium. Endothelial cells have been shown to promote insulin expression and β-cell proliferation (Lammert et al., 2001; Nikolova et al., 2006), and could contribute a critical signal for the final differentiation of β-cells from both embryonic and adult stem cells.

Even before all the steps are worked out for converting ES cells to β-cells, they should provide a useful test platform for basic research on pancreas and islet development. Mouse knockout and transgenic studies have provided a long list of genes important for various steps of pancreas development, yet the underlying circuitry through which these genes interact has proven hard to decipher from simple knockout and overexpression studies. A reproducible ES cell differentiation system – even if not pristine enough for clinical use – should permit quick testing of existing hypotheses as well as generation of new ones. Critical insights gained in ES cells can then be definitively tested in vivo, establishing a virtuous circle in which data from one system informs experiments in the other.

7. Conclusions

The past several years have seen a wealth of new data on the origin of differentiated cells in the embryonic and adult pancreas, and we have been obliged to revise our opinions about pancreatic stem cells and β-cell neogenesis accordingly. Prior to the widespread use of genetic lineage-tracing, we were inclined to accept histological evidence of neogenesis, particularly following injury (Murtaugh and Melton, 2003). Once lineage-tracing studies revealed that division of pre-existing β-cell could account for both growth and regeneration, however (Dor et al., 2004), our view of neogenesis became more skeptical (Murtaugh, 2007). Today, with the recent evidence that pancreatic duct ligation can induce bona fide, *Neurog3*-dependent islet neogenesis (Xu et al., 2008), we are left with a more mixed view, as follows:

- The embryo does contain self-renewing, multipotent progenitor cells, co-expressing *Pdx1*, *Ptf1a* and *Cpa1*. These are not classical stem cells, however, as they do not undergo continued self-renewal through adulthood, but differentiate prior to birth (see Figure 1). Furthermore, when a subset of these cells is destroyed, the others are unable to expand to replace them.
- There is a mechanism to replace lost β-cells in the adult, but it depends primarily on expansion of pre-existing β-cells rather than differentiation of stem or precursor cells (see Figure 3A). A similar expansionist mechanism seems to be in place for acinar cells as well.
- When the exocrine pancreas is injured by duct ligation, a remarkably rapid and robust process of neogenesis is initiated, in which *Neurog3*-expressing islet precursors arise in the ducts and differentiate into new β-cells (and other endocrine cell types, Figure 3B). This response is not quite the same as what we typically call "regeneration": duct ligation does not destroy endogenous β-cells, nor does the animal experience hyperglycemia. Instead, duct ligation seems to evoke the generation of supernumerary β-cells, by mechanisms that remain unknown.

Although the ducts currently appear to be the source of new β-cells following duct ligation, it is unclear if they contain a small number of dedicated stem cells, held in emergency reserve, or if the abnormal environment induces facultative progenitor-like behavior in otherwise normal ducts. Determining whether all duct cells are potential islet precursors, and identifying the signals governing their behavior, will be critical to any clinical use of ducts as a source of new β-cells. Such studies will require the creation of transgenic mice in which mature duct cells can be marked and traced, the absence of which now leaves a major gap in the field. The signals that promote islet development in utero are also poorly understood, and their further characterization should improve efforts to differentiate β-cells from ES cells. We are hopeful that further dialogue between embryology, regeneration and stem cell biology will lead to insights that cross disciplines, and promote the goal of producing islet cells useful for treatment of patients.

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CHAPTER 3

EXOCRINE-TO-ENDOCRINE DIFFERENTIATION IS DETECTABLE ONLY PRIOR TO BIRTH IN THE UNINJURED MOUSE PANCREAS

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Exocrine-to-endocrine differentiation is detectable only prior to birth in the uninjured mouse pancreas

Daniel Kopinke, L Charles Murtaugh*

Abstract

Background: Histological evidence suggests that insulin-producing beta (β)-cells arise in utero from duct-like structures of the fetal exocrine pancreas, and genetic lineage tracing studies indicate that they are maintained in the adult by self-renewal. These studies have not addressed the origin of the new β-cells that arise in large numbers shortly after birth, and contradictory lineage tracing results have been published regarding the differentiation potential of duct cells in this period. We established an independent approach to address this question directly.

Results: We generated mice in which duct and acinar cells, comprising the exocrine pancreas, can be genetically marked by virtue of their expressing the mucin gene *Muc1*. Using these mice, we performed time-specific lineage tracing to determine if these cells undergo endocrine transdifferentiation in vivo. We find that *Muc1*$^+$ cells do give rise to β-cells and other islet cells in utero, providing formal proof that mature islets arise from embryonic duct structures. From birth onwards, *Muc1* lineage-labeled cells are confined to the exocrine compartment, with no detectable contribution to islet cells.

Conclusions: These results argue against a significant contribution by exocrine transdifferentiation to the normal postnatal expansion and maintenance of β-cell mass. Exocrine transdifferentiation has been proposed to occur during injury and regeneration, and our experimental model is suited to test this hypothesis in vivo.

Background

The origin of pancreatic islet cells has been the subject of study and controversy since before the discovery of insulin [1-3]. Histological and ultrastructural studies suggested that islets arise from exocrine ducts during embryogenesis, but whether such exocrine-endocrine conversion continued after birth remained a matter of controversy [4]. Nucleotide analogues have recently been used to identify and trace the fate of proliferating cells, but these studies have been interpreted both for and against the hypothesis of new islet cell differentiation, or neogenesis, in adulthood [5,6]. Similarly, cell culture studies have provided evidence for and against neogenesis, but extrapolating these findings in vivo remains a challenge [2]. Nonetheless, determining whether β-cell neogenesis occurs in vivo will inform efforts to replenish β-cells lost in diabetes: if neogenesis can occur in mice, it might be possible in the human organ as well. Evidence against neogenesis would encourage more aggressive efforts elsewhere, such as the derivation of β-cells from human embryonic stem cells.

Genetic lineage tracing techniques have transformed our understanding of pancreas developmental biology, providing insights either unavailable from or contradictory to prior studies of fixed tissue [2]. For example, using the Cre-loxP system to monitor the fate of cells expressing the acinar protein *Carboxypeptidase A1* (*Cpa1*) has revealed that these cells comprise a self-renewing, multipotent progenitor pool during mid-embryogenesis [7]. At later stages, *Cpa1*$^+$ cells become restricted to the acinar compartment, and lineage tracing of adult acinar cells indicates that they do not contribute to β-cells [7,8]. Other lineage tracing studies similarly cast doubt on the neogenesis model; for example, it is now appreciated that all islet cell types arise

* Correspondence: murtaugh@genetics.utah.edu
University of Utah, Department of Human Genetics, Salt Lake City, UT 84112, USA

from *Neurog3*$^+$ precursor cells [9], yet *Neurog3*$^+$ cells are usually not detected after birth [10-12]. Similarly, a key study of adult β-cell expansion and self-renewal suggests that these processes reflect division of existing β-cells rather than neogenesis [13], a conclusion reinforced by subsequent independent findings [5,14].

Nonetheless, without a Cre line capable of marking duct cells, these studies could not exclude a minor ductal contribution to β-cells. Furthermore, they have not addressed the rapid expansion of β-cell mass that occurs shortly after birth [15], which may derive in part from neogenesis [6]. The ideal tool to address these questions would be a mouse line in which ducts can be inducibly marked at a specific time point, and their ability to contribute to β-cells determined at later stages: a cellular "pulse-chase" experiment [13]. Such mice could also clarify the origin of new β-cells formed in regeneration models, such as duct ligation and partial pancreatectomy, which may involve neogenesis [16,17]

Four transgenic mouse lines have recently been described in studies aimed at addressing this issue. Inada et al. [18] generated mice in which either Cre or its tamoxifen (TM)-inducible derivative CreER™ is driven by the promoter of *Carbonic anhydrase II* (*CAII*), a marker of adult duct cells. This study concluded that ducts continue to give rise to islets after birth, and that neogenesis is dramatically increased after duct ligation [18]. Means et al. [19] used a knock-in approach to target a similar tamoxifen-inducible CreERT molecule to the duct-specific *cytokeratin-19* (*K19*) gene. $K19^{CreERT}$ labels many duct cells in neonatal and adult mice, as well as a small fraction of islet cells due to "leaky" recombinase expression in islets themselves. When $K19^{CreERT}$ was activated by TM treatment at birth, islet labeling at one week of age was no greater than expected from this background activity, suggesting that new islet cells generated in the interim did not derive from the more robustly-labeled duct population [19]. Finally, Solar et al. [20] generated a BAC transgenic in which TM-dependent CreERT2 is targeted to the first exon of *Hnf1β*, a transcription factor expressed selectively in embryonic and adult duct cells. This study indicated that *Hnf1β*$^+$ duct cells give rise to β cells prior to birth but not thereafter, even in the context of injury models such as duct ligation [20]. The apparent contradiction between these studies, in particular those using *CAII*- and *Hnf1β*-driven Cre transgenes, suggests that additional markers should be sought out and exploited for their capacity to label duct cells, thereby providing independent evidence for or against postnatal neogenesis [21].

The gene *mucin1, transmembrane* (*Muc1*) is expressed throughout the ductal network in both the embryonic and adult pancreas, and excluded from islets [22,23]. We have generated mice in which the *Muc1* gene is tagged with an *IRES-CreERT2* cassette, permitting the inducible labeling of *Muc1*$^+$ cells. We find that this line also marks embryonic and postnatal acinar cells, reflecting endogenous Muc1 expression, but is completely excluded from islets in short-term "pulse" experiments. In the embryo, *Muc1*$^+$ cells give rise to endocrine cells as well as their *Neurog3*$^+$precursors, formally confirming that islets originate from fetal ducts. Following birth, however, we find that the *Muc1*$^+$ lineage completely fails to contribute to new islet α- or β-cells, indicating that both the expansion and homeostasis of these cell types occurs independent of contribution from exocrine ducts or acini. These results argue against a major role for neogenesis in the normal postnatal pancreas, and set the groundwork for studies of potential neogenesis during regeneration.

Results

Targeting CreERT2 to the *Muc1* locus

To determine if the *Muc1* locus could be exploited to mark ducts, we evaluated its expression in the embryonic and adult pancreas. We find that Muc1 is widely expressed at E11.5, when most pancreatic cells are still undifferentiated progenitors (Figure 1A-C). As embryogenesis proceeds, Muc1 expression persists in the branching epithelial network (Figure 1D-F), and it is expressed by all ductal cells of the mature organ (Figure 1G-I). Another duct marker, cytokeratin-19 (CK19), is undetectable prior to E17.5 (Figure 2). Interestingly, Muc1 expression appears to decrease as duct caliber increases, opposite to CK19 (Figure 2G-I). Nonetheless, we have not observed, at any stage, a Muc1-negative cell incorporated within a duct structure.

Embryonic islet precursors express the transcription factor *Neurogenin3* (*Neurog3*), and are considered to arise from primitive duct-like cells [4,9-11]. We find that Neurog3 expression localizes within or in close proximity to Muc1$^+$ cells at E15.5, consistent with a ductal origin for islet cells (Figure 3A). While Neurog3 expression is not detectable in adults, Muc1 remains expressed throughout the ductal network of the pancreas, including large ducts as well as fine terminal branches within acini (Figure 3B). Importantly, Muc1 expression is excluded from islet cells during embryogenesis as well as in the mature organ (Figs. 1, 3C). Altogether, *Muc1* appears to satisfy our requirements for a Cre driver line to study islet neogenesis: it is expressed in all duct cells, embryonic and adult, and excluded from differentiated islets.

As detailed in the Methods, we generated a $Muc1^{IRES\text{-}CreERT2}$ allele (henceforth, $Muc1^{IC2}$) by gene targeting, introducing an *IRES-CreERT2* cassette after the endogenous stop codon of the Muc1 locus (Figure 3D-E).

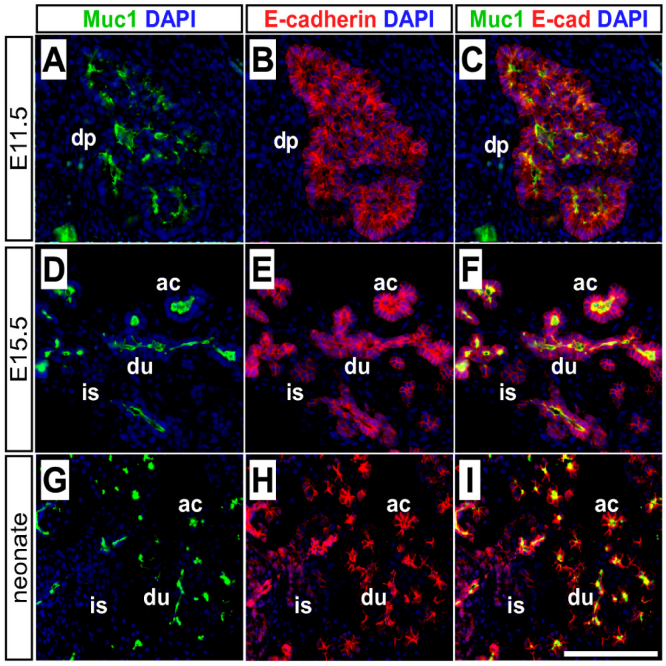

Figure 1 Muc1 expression during pancreas development. Double-immunofluorescence was performed to detect Muc1 (green) and the pan-epithelial marker E-cadherin (red) in sections of embryonic and neonatal pancreata. Muc1 is already strongly expressed in the E11.5 pancreatic epithelium (A-C), when most cells are still undifferentiated progenitors. From E15.5 (D-F) through birth (G-I) Muc1 expression remains strong and becomes restricted to cells lining ductal (du) and acinar (ac) lumens. Islet cells (is) do not express Muc1 at any stage. Scale bar: 100 μm. Abbreviations: ac, acinus; du, duct; is, islet.

The CreERT2 protein is a tamoxifen (TM)-dependent recombinase that provides temporal control of Cre activity [24], which should allow us to investigate the differentiation potential of embryonic and postnatal duct cells. *Muc1*^{IC2} heterozygous and homozygous mice are viable and fertile (data not shown).

Muc1^{IC2} marks exocrine cells in the adult pancreas

To determine whether *Muc1*^{IC2} marks ducts, we performed short-term labeling experiments with the Cre-dependent EYFP reporter strain, *Rosa26*^{EYFP} [25]. We induced recombination by treating adult (6-8 week-old, n = 3) *Muc1*$^{IC2/+}$;*Rosa26EYFP*$^{/+}$ mice with a single dose of 10 mg tamoxifen. After a two-day "chase" period, we found the EYFP lineage label not only in cytokeratin-19^{+} duct cells (~6%), as expected, but also within amylase^{+} acinar cells (Figure 4A-E, ~12%), indicating that this Cre line is active in both duct and acinar cells. (Note that in this and other experiments, we find that *Rosa26*^{EYFP} appears to drive stronger EYFP expression in acinar cells than ducts.) An identical labeling distribution was observed using a different reporter strain, *Rosa26*^{LacZ} [26] (data not shown). In these and other experiments, all *Muc1*^{IC2}-labeled cells were found to be E-cadherin^{+} (data not shown), indicating that they represent pancreatic parenchymal cells rather than connective tissue or vasculature.

Having observed the lineage label in acinar cells, we wanted to confirm that these cells actually express Muc1. After enzymatic dissociation and immunostaining of isolated acini, we detect Muc1 not only within small acini, comprised entirely of amylase^{+} cells, but also in single amylase^{+} cells (Figure 5A-F). Confocal imaging of wholemount stained pancreata confirmed that Muc1 protein localizes to the apical poles of acinar cells, and is readily detected within individual acini that lack

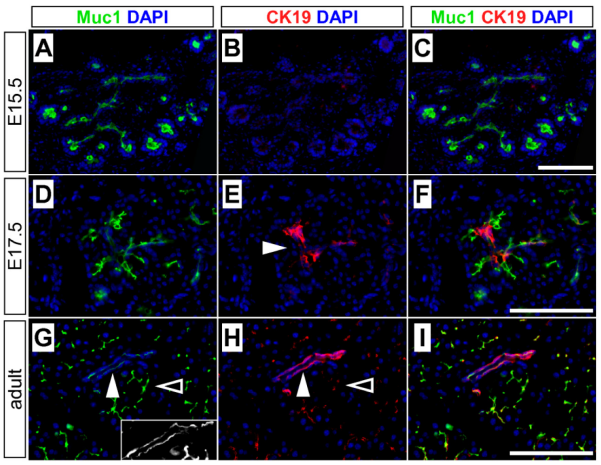

Figure 2 Comparison of Muc1 and CK19 staining. Double-immunofluorescence was performed to detect Muc1 (green) and cytokeratin-19 (CK19, red) in sections of embryonic and adult pancreata. (A-C) At E15.5, CK19 is undetectable while Muc1 is strongly expressed throughout the pancreatic ductal epithelium. (D-F) CK19 expression is first detected in larger ducts at E17.5, but remains weak or undetectable in smaller ones, while ducts of all sizes express Muc1. (G-I) In the adult pancreas, Muc1 protein is detected in all ducts, albeit at slightly lower levels in intralobular (closed arrowhead) than intercalated ducts (open arrowhead). (Insert in G demonstrates that Muc1 is expressed by all duct cells.) CK19 exhibits the opposite staining pattern, highest in intralobular and lowest in intercalated ducts. Scale bars: 100 μm.

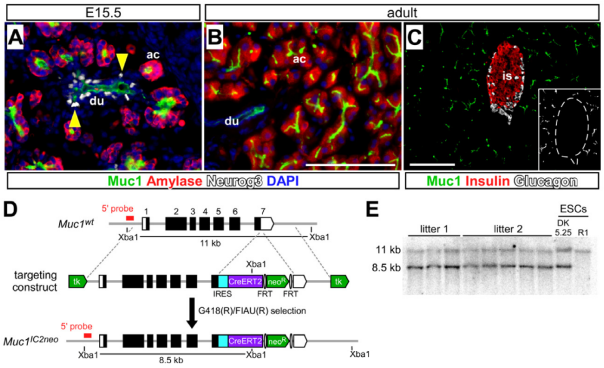

Figure 3 Muc1 expression and gene targeting. (A) At E15.5, Muc1 (green) is expressed by the ductal core, in which Neurog3^{+} islet precursors (white, indicated by yellow arrowheads) appear to reside, as well as within the terminal elements of the epithelial network, adjacent to amylase-expressing acini (red). (B) While Muc1 remains strongly expressed throughout the adult ductal tree, Neurog3 is not detectable. (C) Muc1 (green) is not detected within or adjacent to adult islet β-cells (insulin, red) or α-cells (glucagon, white). Inset depicts Muc1 staining alone (white), with islet boundaries indicated by dashed line. (D) Structure of *Muc1* locus, targeting vector and targeted allele. The targeting vector was designed to introduce an *IRES-CreERT2* cassette downstream of the Muc1 stop codon, along with a FRT-flanked *neo*^{R} construct for positive G418 selection and a *tk* gene at the end of each homology arm for negative FIAU selection in ES cells. Dotted lines indicate the boundaries of the 5' and 3' homology arms used for targeting, and the probe used for Southern blotting (outside the 5' homology arm) is indicated in red. Correct targeting introduces a new XbaI site, shifting the predicted restriction fragments detected by this probe. (E) Southern blotting of *Muc1*^{IC2neo} ES cells and mice. Xba1 digests of genomic DNA prepared from ES cells (parental R1 cells or targeted clone DK5.25), as well as from putative *Muc1*$^{IC2neo/+}$ F1 offspring of DK5.25 chimeras (genotyped by PCR), were hybridized with the probe indicated in panel A. Targeted ES cells and mice display both wildtype (11 kb) and *IC2neo* (8.5 kb) XbaI fragments. Scale bars: 100 μm. Abbreviations: ac, acinus; du, duct; is, islet.

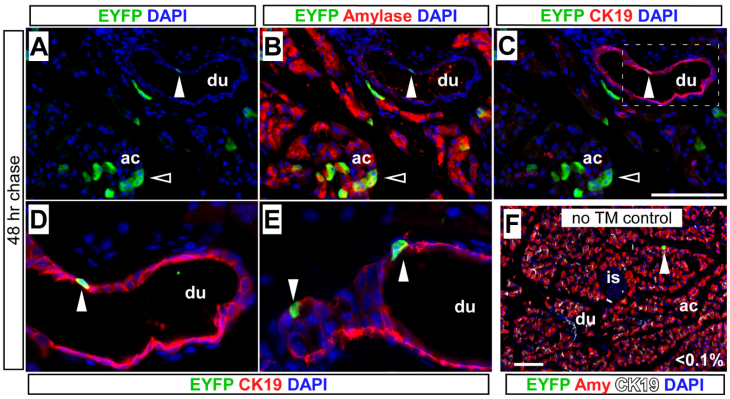

Figure 4 *Muc1*$^{IRES\text{-}CreERT2}$ is active in adult exocrine cells. (A-C) Recombination was induced in adult (P60) *Muc1*$^{IC2/+}$;*Rosa26*$^{EYFP/+}$ mice by administration of 10 mg tamoxifen, and mice were analyzed 2 days later by immunofluorescence for EYFP (green), amylase (B, red) and cytokeratin-19 (C, red), together with DAPI (blue) to mark nuclei. After a short term chase, *Muc1*^{IC2} marks cells of the ductal network (closed arrowheads) as well as acinar cells (open arrowheads), suggesting that Muc1 is expressed in both duct and acinar cells. (D-E) Ductal *Rosa26*^{EYFP} labeling by *Muc1*^{IC2}, shown in fields devoid of labeled acinar cells (D, magnified from panel C; E, independent field). As *Rosa26*^{EYFP} expression is lower in duct cells than acini, we have increased the green signal in these panels to make ductal staining more obvious. (F) 8-month old TM-untreated control mouse, stained for EYFP (green), amylase (red) and cytokeratin-19 (white). Less than 0.1% of duct and acinar cells are labeled in the absence of TM. Scale bars: 100 μm. Abbreviations: ac, acinus; du, duct; is, islet.

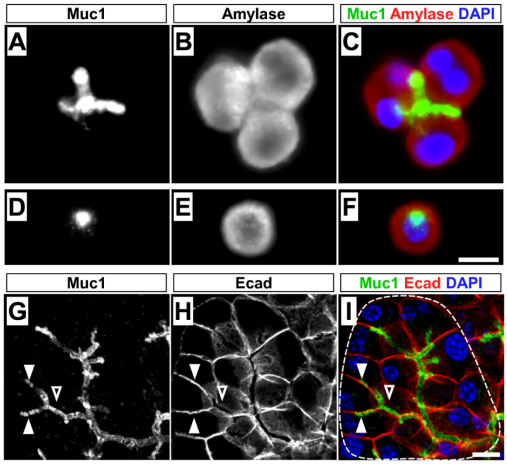

Figure 5 Muc1 localization within acinar structures. (A-F) Immunofluorescence of dissociated wildtype acini for Muc1 (green), amylase (red) and DAPI (blue). Muc1 is detected within small clusters of amylase^{+} acinar cells as well as in single amylase^{+} cells, suggesting that these cells, as well as ducts, express the *Muc1* gene. (G-I) Confocal z-projection (0.75 μm) of adult wildtype pancreas, wholemount stained for Muc1 (green), E-cadherin (red) and DAPI (blue). Outline in I indicates a single acinar unit, with a shared central lumen. Membrane-bound Muc1 protein localizes to this central lumen (open arrowhead), as well as extending between two individual acinar cells (closed arrowhead). Note that there appears to be no centroacinar cell in this cluster, the lumen of which is entirely defined by the apical surfaces of adjacent acinar cells, and coated by Muc1 protein. Scale bars: 10 μm.

centroacinar cells [27] (Figure 5G-I). Since this antibody recognizes the intracellular domain of the Muc1 protein [28], the observed staining is unlikely to reflect shed or contaminating Muc1 from duct or centroacinar cells. In situ hybridization studies have similarly revealed *MUC1* expression in both acinar and duct cells of the human pancreas [29], and we conclude that the acinar labeling observed with $Muc1^{IC2}$ reflects endogenous *Muc1* expression.

We also determined the relative distribution of $Muc1^{IC2}$-labeled cells between small (intercalated), medium (intralobular) and large (interlobular) ducts (Figure 6A-C). We found that $Muc1^{IC2}$-labeled cells were present in all three duct types, with slightly lower labeling within interlobular ducts than those of smaller caliber (Figure 6D). This labeling distribution is consistent with the distribution of Muc1 protein itself, described above (Figure 2), and suggests that $Muc1^{IC2}$-driven recombination can occur in any *Muc1*-expressing cell.

Examining TM-untreated 4-8 month $Muc1^{IC2/+}$; $Rosa26EYFP^{/+}$ and $Muc1^{IC2/+}$;$Rosa26LacZ^{/+}$ mice (n = 3) revealed a background recombination rate of less than 0.1% (Figure 4F and data not shown), confirming that this line exhibits stringent tamoxifen dependence. Furthermore, we never observed lineage-labeled endocrine cells in our short-term experiments. $Muc1^{IC2}$ labeling is thus TM-dependent, as expected, and extends throughout the exocrine pancreas (acini and ducts). Although TM-induced $Muc1^{IC2}$ labeling is relatively sparse (5-15% of exocrine cells, in this experiment), its distribution closely matches that of Muc1 itself, and appears to represent a random sampling of the exocrine pancreas. As $Muc1^{IC2}$ does not directly label islet cells, it can be used to detect potential exocrine-derived neogenesis.

Islet cells arise from embryonic *Muc1*$^+$ cells

To determine whether $Muc1^+$ cells contribute to islet neogenesis in utero, we administered tamoxifen to pregnant females carrying $Muc1^{IC2/+}$; $Rosa26EYFP^{/+}$ embryos. These experiments yielded a very low overall labeling rate (≤1% of any cell type, data not shown), possibly due to reduced amounts of TM entering fetal circulation. To increase the cellular Cre concentration, therefore, we used homozygous $Muc1^{IC2/IC2}$; $Rosa26EYFP^{/+}$ embryos for all in utero labeling experiments. $Muc1^{IC2/IC2}$;$Rosa26EYFP^{/+}$ embryos were labeled with a single maternal dose of TM (5-10 mg) at different embryonic stages: E11.5, E13.5 or E15.5. All embryos were analyzed at E17.5 (n = 3 for each tamoxifen treatment group). As expected, $Muc1^{IC2}$ labels fetal ducts at all these timepoints (Figure 7A and data not shown), albeit at low frequencies (2-6%, Figure 7B). Consistent with its labeling pattern in the adult pancreas, $Muc1^{IC2}$ marks a similar proportion of fetal acinar cells as well (Figure 3B and data not shown). Furthermore, we observe EYFP lineage-labeling of insulin$^+$ β-cells and glucagon$^+$ α-cells in all of these pancreata (Figure 7B-D), indicating an origin within the $Muc1^+$ exocrine compartment. Our experimental design therefore identifies potential neogenesis even from a sparsely-labeled population. As β-cell numbers increase exponentially between E11.5 and E17.5 [30], ~100-fold total, most of the β-cells analyzed in this experiment would have been born since tamoxifen was administered. That we find comparable $Muc1^{IC2}$ lineage labeling in β-cells and exocrine cells (Figure 7B) suggests that most of the new β-cells arose via exocrine-derived neogenesis.

To further confirm that $Muc1^{IC2}$ is not active in fetal islets themselves, we performed short-term (12-14 hr) pulse-chase labeling of embryos at E13.75 or E14.75. We could easily detect the lineage label in the embryonic ductal system as well as in Neuog3$^+$ cells (Figure 8A-B), but failed to detect any labeled hormone-producing cells (Figure 8C-D). The short-term labeling of Neurog3$^+$ cells agrees with the co-expression of Neurog3 and Muc1 in embryonic ducts (Figure 3A), and the lack of short-term islet labeling confirms that the later appearance of labeled endocrine cells reflects differentiation from exocrine tissue.

Finally, to determine whether fetal $Muc1^+$ cells contribute to adult islets, we administered 5 mg TM to pregnant females at E14.5 and analyzed labeling at weaning age (P21). We found labeling of both β-cells (2.2 +/- 0.4%) and α-cells (2.1% +/- 0.2%), present in mature islet structures (Figure 9). As expected, lineage label also persists in adult duct cells, and appears to be distributed equally among various classes of duct structures (Figure 6D).

Altogether, these results strongly suggest that (a) embryonic $Muc1^+$ duct cells give rise to all segments of the adult ductal network, (b) *Muc1* is not expressed by endocrine cells and (c) mature islet cells arise from *Neurog3*$^+$ precursors within the embryonic $Muc1^+$ ductal network. These results represent direct evidence that endocrine cells arise from embryonic ducts, but they leave open the possibility that embryonic $Muc1^{IC2}$ labeling actually marks a ductal stem cell-like population, which continues to give rise to new islet cells after birth. We therefore turned our attention to the differentiation potential of postnatal $Muc1^+$ cells.

Muc1$^+$ exocrine cells do not undergo endocrine differentiation after birth

We performed three experiments to determine whether the $Muc1^+$ exocrine compartment contributes to islet cells after birth. In experiment 1, we administered 10 mg tamoxifen to young adult (~P60) $Muc1^{IC2/+}$;

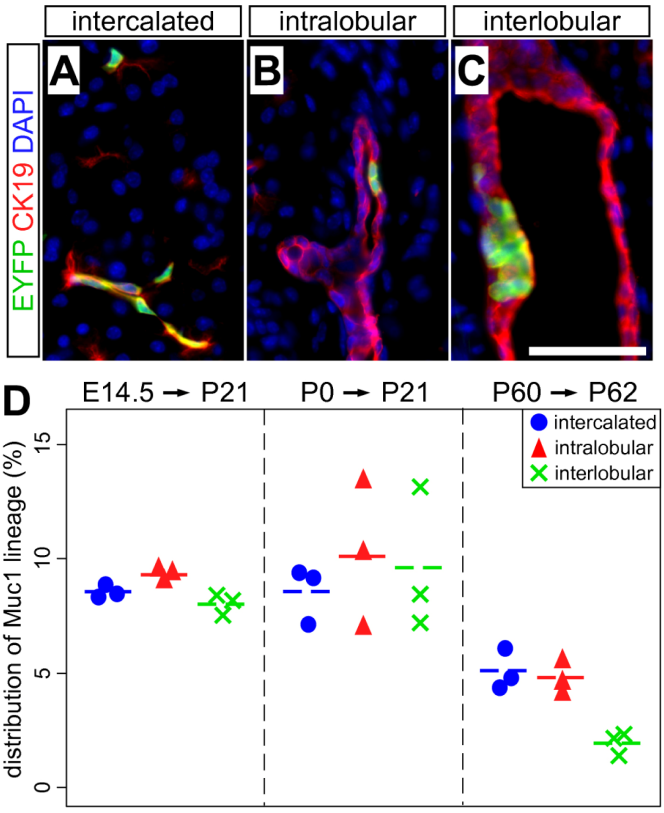

Figure 6 *Muc1*^{IC2} labels cells throughout the ductal network. (A-C) Representative immunofluorescence images documenting the presence of *Muc1*^{IC2}-labeled cells (green) within small/intercalated, medium/intralobular and large/interlobular ducts, co-stained for cytokeratin-19 (red). Interlobular were distinguished from intralobular ducts by the presence of connective tissue separating them from acinar parenchyma. Scale bar: 50 μm. (D) *Muc1*^{IC2} lineage-labeling indices of different duct populations, within various pulse-chase experiments. In each experiment, we observe roughly equal labeling of intercalated (blue circle), intralobular (red triangle) and interlobular (green X) ducts, with the exception of decreased intercalated duct labeling after tamoxifen treatment of adults ($p < 0.01$ by TukeyHSD).

Rosa26EYFP$^{l/+}$ mice, and assayed the potential contribution of labeled cells to either exocrine or endocrine cells after 7, 60 or 120 days (Figure 10A-C and data not shown). We observed an overall labeling efficiency (i.e. proportion of EYFP$^+$ cells per field, without reference to cell type-specific markers) of ~24% in these experiments, which included ~30% labeling of acinar and ~10% labeling of duct cells (Table 1). As our short-term labeling experiments never revealed detectable lineage marking of islet cells (D.K., unpublished observations), we focused our quantitative analyses on the 60 d and 120 d chase periods. We counted randomly-chosen fields of β-cells and α-cells, scoring separately for number of insulin$^+$ or glucagon$^+$ cells, number of EYFP$^+$ cells, and number of double-positive (hormone$^+$/EYFP$^+$) cells. In fact, after scoring several thousand cells positive for each marker (Table 2), we never observed a single β-cell or α-cell positive for EYFP, suggesting that new islet cells do not arise in significant numbers from adult *Muc1*$^+$ exocrine cells.

To determine if *Muc1*$^+$ cells contribute to the rapid expansion of islet cell numbers after birth [15], we

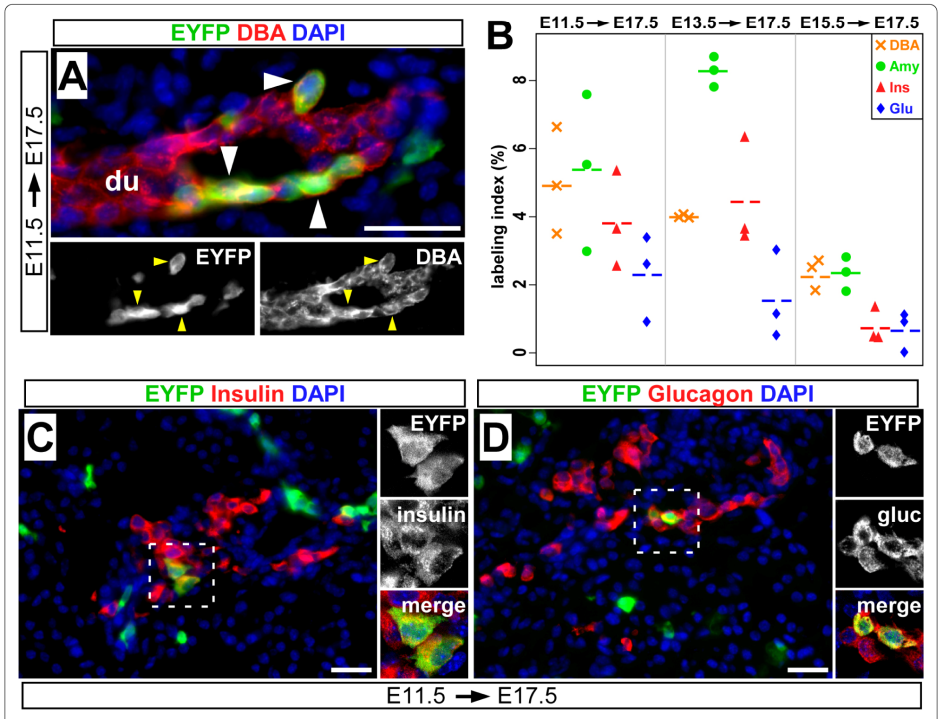

Figure 7 *Muc1*$^{+}$ cells give rise to endocrine cells during embryogenesis. (A) A single dose of 5 mg tamoxifen was given to pregnant mice at E11.5, and pancreata of *Muc1*$^{IC2/IC2}$; *Rosa26*$^{EYFP/+}$ offspring were analyzed at E17.5 by immunofluorescence for EYFP (green), DBA lectin (red) and DAPI (blue). Arrowheads indicate DBA^{+} cells co-expressing EYFP. (B) Quantification of observed co-labeling at E17.5, following tamoxifen administration at indicated time points. Scatter plot depicts the percentages of DBA^{+} (orange), amylase^{+} (green), insulin^{+} (red) and glucagon^{+} (blue) cells co-expressing EYFP. Each point represents an independent embryo, and means are indicated by dotted lines. (C-D) Pancreata of *Muc1*$^{IC2/IC2}$; *Rosa26*$^{EYFP/+}$ embryos, administered TM at E11.5 as in (A), stained at E17.5 for EYFP (green), insulin or glucagon (red) and DAPI (blue). Inserts: confocal z-projection (1.36 μm) of the boxed areas, to confirm localization of EYFP to hormone-producing cells. Scale bars: 25 μm.

performed experiment 2, in which we induced recombination in *Muc1*$^{IC2/+}$; *Rosa26*$^{LacZ/+}$ neonates by administration of tamoxifen to nursing mothers (consecutive 10 mg doses on P0 and P1). We sacrificed mice 21 or 120 days after treatment (i.e. as weanlings or mature adults), and performed immunofluorescence to detect LacZ lineage marker within the exocrine and endocrine compartments (Figure 10D-F and data not shown). We found an overall labeling efficiency of ~10% in these experiments, including ~15% of acinar cells and ~4% of duct cells (Table 1). As in experiment 1, however, despite scoring several thousand cells for each marker, we did not observe a single LacZ^{+} β-cell or α-cell (Table 2).

Previous studies indicate that acinar cells do not contribute to islets after birth [7,8,31], and experiment 2 suggests that neonatal duct cells are also excluded from the islet lineage. This interpretation hinges on relatively infrequent ductal labeling, which could have hidden a low level of duct-derived neogenesis. To increase the duct labeling frequency, we performed experiment 3, in which we directly injected newborn *Muc1*$^{IC2/+}$; *Rosa26EYFP*$^{/+}$ pups with tamoxifen (2 mg per pup, delivered subcutaneously). Upon sacrifice, 21 days after TM administration, we found increased overall labeling compared to mice that received maternal TM (~30% EYFP^{+}, Table 1). Importantly, the duct labeling

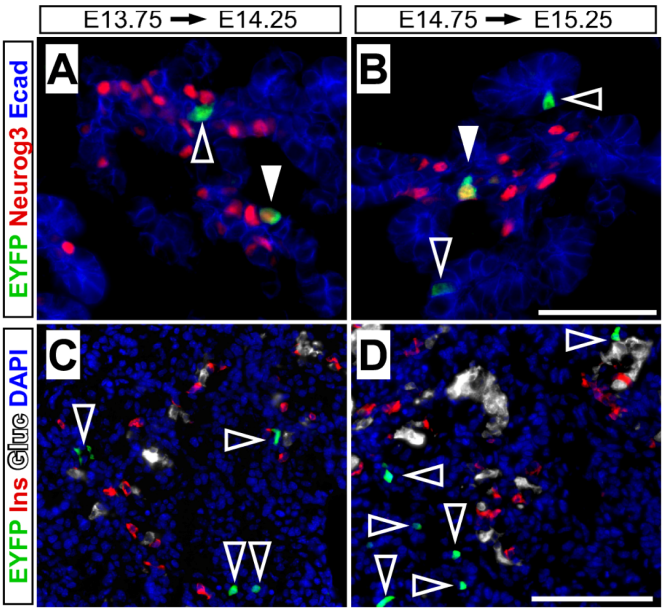

Figure 8 Neurog3$^+$ cells arise from *Muc1*$^+$ ductal cells during embryogenesis. To detect the short-term differentiation potential of *Muc1*$^+$ cells, *Muc1*$^{IC2/IC2}$; *Rosa26EYFP*$^{/+}$ embryos were labeled by TM administration to pregnant dams at E13.75 (A, C) or E14.75 (B, D), and harvested ~12 hours later for immunofluorescence analysis (n = 2 for each timepoint). (A-B) Short-term labeling of *Muc1*$^+$ cells (green) reveals their presence within the E-cadherin$^+$ ductal network (blue, open arrowheads), as well as their contribution to Neurog3$^+$ cells (red, closed arrowheads). (C-D) Similar analysis of islet hormone expression indicates that *Muc1*^{IC2} labeling (green, open arrowheads) is excluded from insulin$^+$ (red) and glucagon$^+$ cells (white), confirming that these cells derive from *Muc1*$^+$ progenitors but do not themselves express *Muc1*. Scale bars: 50 μm in A-B and 100 μm in C-D.

frequency was increased to 10%, with equal distribution among interlobular, intralobular and intercalated ducts (Figure 6D). Nonetheless, we did not observe any labeled β-cells or α-cells, despite scoring several thousand cells positive for each marker (Table 2).

These analyses suggest an upper limit to the contribution of neogenesis to postnatal islet growth. β-cell mass has been reported to expand between 4- and 10-fold in the first 2-4 weeks after birth [32-34]. If we assume a five-fold expansion between P0 and P21, we can infer that ~80% of the β-cells scored in experiment 3 were "new" since P0 (3600 of the ~4500 β-cells counted, Table 2). If all of these had been derived from *Muc1*^{IC2}-labeled duct cells, given a duct labeling index of ~10% (Table 1), we would have expected to observe roughly 360 labeled β-cells. As we observed zero, we conclude that ≤1% of all β-cells generated after birth could have arisen from labeled ducts (1% neogenesis would have resulted in ~4 labeled β-cells, which is probably near the limit of reliable detection). Altogether, experiments 1-3 fail to reveal duct-to-islet transdifferentiation after birth.

Discussion

At birth, the mammalian β-cell changes from a metabolic passenger to the driver of glucose homeostasis. Based on our results and those of Solar et al. [20], we propose that the mechanisms controlling β-cell mass also change at birth, from a fetal period of new differentiation, or neogenesis, to a mature state of self-renewal (Figure 11). To detect this transition, we performed a direct comparison of duct and acinar cell lineages before and after birth. We provide formal proof – confirming prior studies of histology and gene expression – that islets arise from embryonic *Muc1*$^+$ ducts. From birth onwards, however, we find no evidence for a ductal

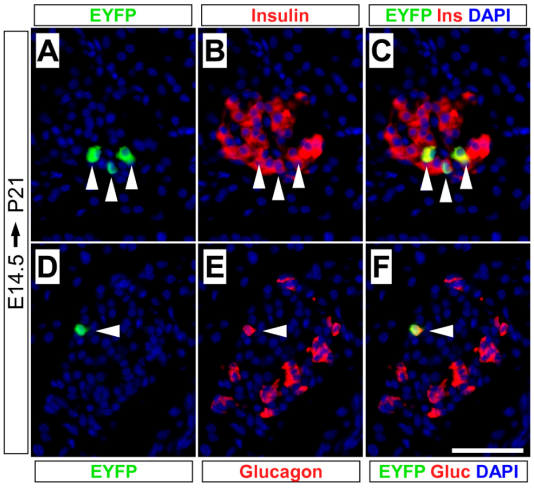

Figure 9 Postnatal islet cells derive from embryonic *Muc1*$^{+}$ progenitors. *Muc1*$^{IC2/IC2}$; *Rosa26*$^{EYFP/+}$ mice received tamoxifen in utero (5 mg to pregnant dam at E14.5), and were analyzed at P21 by immunofluorescence for EYFP lineage label (green), insulin (A-C, red) and glucagon (D-F, red). After inducing the Muc1 lineage at E14.5 and chasing offspring until P21, EYFP labeling (white arrowheads) is detected among both insulin^{+} β-cells (2.2 +/- 0.4%) and glucagon^{+} α-cells (2.1 +/- 0.2%). Scale bar: 50 μm.

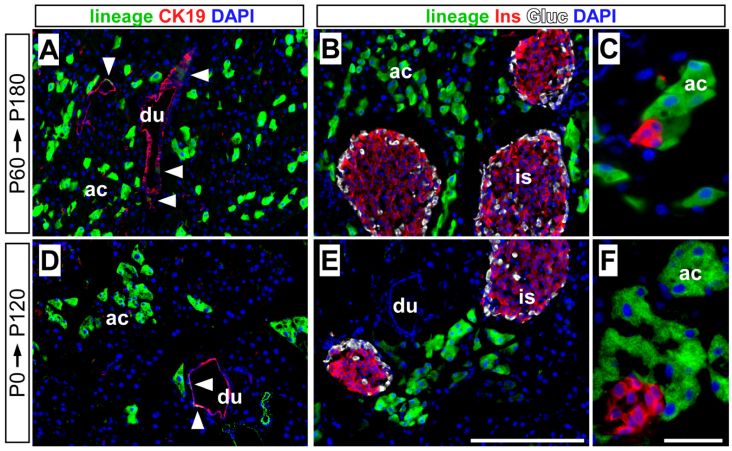

Figure 10 The *Muc1* lineage does not contribute to islets after birth. (A-C) Experiment 1: adult *Muc1*$^{IC2/+}$; *Rosa26*$^{EYFP/+}$ mice (P60) were treated with a single dose of 10 mg tamoxifen, and analyzed after a 120 d chase period (i.e. to six months of age). Immunofluorescence was performed for EYFP lineage label (green), cytokeratin-19 (A, red), insulin (B-C, red) and glucagon (B, white). Lineage label remains restricted to the exocrine compartment (arrowheads indicate labeled duct cells). (D-F) Experiment 2: *Muc1*$^{IC2/+}$; *Rosa26*$^{LacZ/+}$ neonates received TM via maternal gavage (sequential 10 mg doses at P0 and P1), and were analyzed after 120 d by immunofluorescence as per A-C (using LacZ, in green, as lineage label). LacZ labeling is confined to duct (white arrowheads) and acinar cells, with no detectable expression in islet cells. Scale bars: A-B and D-E, 100 μm; C and F, 25 μm. Abbreviations: du, duct cells; ac, acini.

Table 1 *Muc1*^{IC2} lineage contribution to exocrine cells

					Lineage$^+$ (EYFP or LacZ)		
Experiment	Genotype	Pulse protocol	Chase period	Sample #	% of DAPI$^+$	% of AMY$^+$	% of CK19$^+$
1	Muc1$^{IC2/+}$; Rosa26$^{EYFP/+}$	adult (P60) 10 mg TM gavage	7 d	1	23.6	29.3	7.5
				2	22	27.3	6.2
				3	24.3	32.2	5.4
				mean	23.3 ± 0.7	29.6 ± 1.4	6.4 ± 0.6
			120 d	1	30	42.4	8.5
				2	12.6	15	11.7
				3	20.8	26.4	10.1
				4	33	43.6	14.5
				5	23.6	30.6	13.3
				mean	24.0 ± 3.6	31.6 ± 5.3	11.6 ± 1.1
			overall mean		23.7 ± 2.2	30.8 ± 3.2	9.6 ± 1.2
2	Muc1$^{IC2/+}$; Rosa26$^{LacZ/+}$	pups (P0/P1) 10 mg TM maternal gavage	21 d	1	16.6	22.9	9.9
				2	11.7	15.5	3.6
				3	3.3	4.1	1.8
				4	6.2	7.4	1.9
				5	5.1	5.9	1.5
				mean	8.6 ± 2.4	11.2 ± 3.5	3.7 ± 1.6
			120 d	1	14.1	21.9	5.5
				2	21.4	30.9	6.2
				mean	17.7 ± 3.7	26.4 ± 4.5	5.8 ± 0.4
			overall mean		10.6 ± 2.5	15.5 ± 3.8	4.3 ± 1.2
3	Muc1$^{IC2/+}$; Rosa26$^{EYFP/+}$	pups (P0) 2 mg TM SQ	21 d	1	21.7	ND	8.9
				2	22.8		7.8
				3	51.1		13.5
				mean	31.9 ± 9.6		10.1 ± 1.7

Three "pulse-chase" lineage-labeling experiments were performed, as described in the text, in which tamoxifen was administered (by oral gavage to adults, or subcutaneous injection to neonates) and mice sacrificed after chase periods of 7-120 days. For each sample, we counted total cells per field (DAPI), acinar cells (amylase$^+$) and duct cells (cytokeratin-19$^+$), as well as the number of cells double-positive for these markers and for the lineage tracer. From these counts, we derived the labeling efficiency (expressed as a percentage) of total cells (DAPI), acinar (AMY) and duct (CK19) cells. Shown are summary data for every mouse analyzed, as well as mean values +/- standard error. ND, not determined.

origin of new β-cells, and we propose that postnatal β-cell expansion and homeostasis normally occur without contribution from ducts or acini.

We had intended, in creating the *Muc1*^{IC2} allele, to specifically address the differentiation potential of duct cells. Instead, we find that *Muc1*^{IC2} labels both acinar and duct cells, at all stages examined, and that Muc1 protein is readily detected within acinar cells. Nonetheless, we can treat the labeling of postnatal acinar cells as "background," as acinar-to-islet transdifferentiation does not occur after birth [7,8,31]. Cells expressing the acinar enzyme *Cpa1* do behave as multipotent "tip cell" progenitors prior to E13.5, but are thereafter restricted to the acinar lineage [7]. As *Muc1*$^+$ cells still contribute to islets at E13.5 and E15.5 (Figs. 7, 9), we propose that islet differentiation competence normally shifts from *Muc1*$^+$/*Cpa1*$^+$ tips to *Muc1*$^+$/*Cpa1*-negative "trunks" after E13.5, before being lost entirely at birth (Figure 11).

Another recently developed mouse line, *K19*^{CreERT}, in which CreERT is targeted to the *cytokeratin-19* locus, drives TM-dependent recombination in inter- and intralobular ducts [19]. Unlike *Muc1*^{IC2}, *K19*^{CreERT} does not label distal intercalated ducts, and is active in a small fraction of islet cells. Nonetheless, preliminary experiments reported using *K19*^{CreERT} provide independent evidence supporting our model: TM treatment at birth

Table 2 Quantification of potential Muc1 contribution to endocrine cells

					number of cells scored		
Experiment	Genotype	Pulse protocol	Chase period	Sample #	lineage$^+$	INS$^+$	GLU$^+$
1	Muc1$^{IC2/+}$; Rosa26$^{EYFP/+}$	adult (P60) 10 mg TM gavage	60 d	1	2950	2054	ND
				2	959	1398	
				total	3909	3452	
			120 d	1	1588	624	209
				2	1416	498	144
				3	1266	1069	374
				4	2089	2004	703
				5	1199	1488	409
				6	1079	904	369
				total	8637	6587	2208
2	Muc1$^{IC2/+}$; Rosa26$^{LacZ/+}$	pups (P0/P1) 10 mg TM maternal gavage	21 d	1	221	590	276
				2	483	745	235
				3	266	1045	436
				total	970	2380	947
			120 d	1	2072	2152	626
				2	2637	1847	315
				total	4709	3999	941
3	Muc1$^{IC2/+}$; Rosa26$^{EYFP/+}$	pups (P0) 2 mg TM SQ	21 d	1	1566	2017	999
				2	1264	950	488
				3	3508	1570	564
				Total	6338	4537	2051

As described in the text, mice subjected to "pulse-chase" labeling with *Muc1*^{IC2} were analyzed for potential contribution of lineage-marked cells to insulin$^+$ β-cells or glucagon$^+$ α-cells (exocrine labeling results in Table 1). Indicated are the total number of cells scored as positive for lineage label (EYFP or LacZ), as well as the total number of insulin$^+$ and glucagon$^+$ cells scored in the same fields. In no case did we observe a lineage label-positive endocrine cell. ND, not determined.

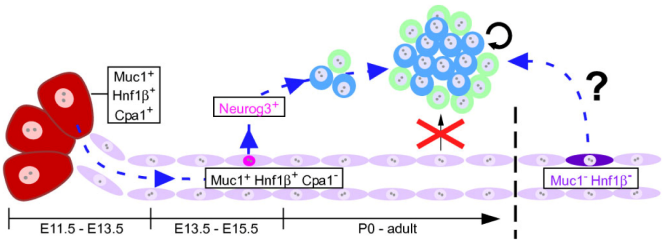

Figure 11 Dynamic differentiation potential within theexocrine pancreas. Multipotent pancreatic progenitors (E11.5-E13.5) express the digestive enzyme Cpa1, which is later restricted to acinar cells (E14.5-adult), together with Muc1 and Hnf1β [20]. While *Cpa1*$^+$ cells cease contribution to islets from approximately E13.5 [7], *Muc1*$^+$/*Hnf1β*$^+$ cells continue to give rise to *Neurog3*$^+$ endocrine precursors (pink nuclei) and islets (blue and green) through at least E15.5. From around birth, *Muc1*$^+$ and *Hnf1β*$^+$ cells no longer contribute to the endocrine lineage, and mature islets are maintained by self-replication of those pre-existing. However, it remains a formal possibility that a subpopulation exists within the ductal network (dark blue cytoplasm), expressing neither *Muc1* nor *Hnf1β*, from which β-cells continue to arise after birth.

results in ≥10% labeling of ducts after one week, but <1% labeling of islets, equivalent to the direct activity of this line in islet cells themselves [19].

While this manuscript was in preparation, Solar and colleagues [20] published a study using another exocrine CreERT2 line, driven by the *Hnf1β* locus. Unlike $Muc1^{IC2}$, this driver is not active in acini, and labels a higher fraction of duct cells postnatally (approximately 20% at birth and 40% in adults, compared to 10% labeling at either timepoint with $Muc1^{IC2}$). As with $Muc1^{IC2}$, lineage-tracing of $Hnf1\beta^{+}$ cells revealed duct-to-islet differentiation prior to birth, but none thereafter. Further experiments by these investigators indicate that such differentiation does not occur in the context of injury and regeneration [20], as previously believed [16]. Our data provide further evidence against postnatal duct-to-islet differentiation in the healthy pancreas, although it remains to be determined if injury can induce neogenesis from $Muc1^{IC2}$-expressing population.

The *Hnf1β-CreERT2* and $Muc1^{IC2}$ lineage tracing results contradict those obtained with a Cre transgene driven by the *Carbonic anhydrase II* promoter (*CAII-Cre*) [18]. Using $Rosa26^{LacZ}$ reporter mice to detect recombination [26], these authors report that *CAII-Cre* drives duct-restricted recombination beginning at E18.5, but labels roughly 15% of β-cells at four weeks of age. We cannot offer an obvious explanation for this discrepancy; given the number of β-cells that we counted, we should have detected such a robust contribution from $Muc1^{IC2}$-labeled duct cells. One possibility is that *CAII-Cre*-catalyzed recombination actually begins prior to birth, when $Neurog3^{+}$ cells are still present [10,11], but that LacZ expression cannot be detected until one or more days after birth. In fact, half of the newborn pancreata examined in this study already exhibited at least some labeled β-cells (in one pup, as many as 70% of islets contained labeled β-cells), indicating prenatal recombination [18]. These authors have also generated a *CAII-CreERT* transgene, which could be used to follow postnatal labeling specifically, although their experiments with these mice revealed surprisingly high levels of tamoxifen and Cre-independent LacZ expression in adult islets [18].

Alternatively, a subpopulation of duct cells with the capacity for islet differentiation might escape labeling by $Muc1^{IC2}$, $K19^{CreERT}$ and *Hnf1β-CreERT2*, but not by *CAII-Cre* (Figure 11). Indeed, as neither our Cre driver nor those described by others labels a majority of postnatal duct cells [19,20], the possibility of a substantial unmarked subpopulation is impossible to exclude formally. As three independent and distinct Cre transgenes have yielded identical conclusions, however, the burden of evidence would appear to weigh against postnatal islet neogenesis.

With respect to $Muc1^{IC2}$ in particular, we note that although its recombination efficiency in utero is even lower than after birth, our experimental approach still identifies islet cells arising from the sparsely labeled embryonic exocrine compartment. Furthermore, we have never observed a Muc1-negative duct cell (Figs. 1, 2, 3, and data not shown), nor is there evidence for anatomical exclusion of $Muc1^{IC2}$-labeled cells within the ductal network (Figure 6). We also do not observe any obvious change in Muc1 expression or distribution between embryonic stages, when $Muc1^{IC2}$ does label endocrine cells, and postnatal stages, when it does not. An obvious transition that does occur perinatally is the extinction of *Neurog3* expression, which itself weighs against the persistence of duct-to-islet differentiation after birth [10,11].

From a physiological perspective, it might make sense that expansion of β-cells after birth involves a mechanism independent of neogenesis, as postnatal β-cells must contend with metabolic demands from which embryonic progenitor cells are buffered. In fact, numerous knockout mouse studies indicate the existence of postnatal-specific mechanisms to control β-cell mass [32,35-39]. Furthermore, recent studies suggest that expansion of β-cell mass in adults, in response to β-cell damage or increased insulin demand, occurs via proliferation rather than neogenesis [13,37,40]. Potential exceptions to this rule have been described, including partial pancreatectomy and duct ligation, in which development of new β-cells is accompanied by the re-appearance of *Neurog3*-expressing cells within the ductal epithelium [16,17]. Studies using *Hnf1β-CreERT2* to mark pre-existing duct cells in such models did not detect contribution to new β-cells, however [20]. The $Muc1^{IC2}$ line is well suited for similar experiments and, as a tool to mark cells throughout the exocrine pancreas, it should complement and extend results obtained by others.

Conclusions

Our results constitute formal evidence that insulin-producing β-cells, and other endocrine cells of the mature pancreatic islet, derive from ductal cells of the embryonic organ. Furthermore, the ability to trace the lineage of cells expressing *Muc1* at different timepoints allows us to compare their differentiation potential before and after birth. We find that *Muc1*-expressing cells lose the capacity for islet differentiation postnatally, prior to the major increase in β-cell numbers that occurs in juvenile mice. These data add to an emerging model for control of β-cell mass, driven by developmentally-programmed neogenesis in the womb and physiologically-regulated proliferation after birth.

Methods

Targeting CreERT2 to the *Muc1* locus

We followed the procedure of Wu et al. [41] to generate a $Muc1^{IRES\text{-}CreERT2\text{-}neo}$ ($Muc1^{IC2neo}$) targeting vector. In brief, we recombineered a 9.2 kb fragment of the mouse *Muc1* gene from a 129Sv BAC library (clone bMQ-356N19, from the Sanger Institute) into a conventional plasmid, introduced an *IRES-CreERT2-FRT-neo*^{R}*-FRT* cassette after the endogenous Muc1 stop codon, and flanked the homology arms with *thymidine kinase* (*tk*) cassettes (Figure 3D). The targeting vector was then electroporated into R1 ES cells [42], generously provided by Mario Capecchi, which were selected with G418 and FIAU [43]. 52/96 of the surviving clones exhibited homologous recombination upon Southern blotting with a probe located outside the 5' homology arm. Proper recombination was confirmed for 8/8 of these upon further Southern analyses, and one of these clones, DK5.25 (Figure 3E), was used to generate chimeras (University of Utah, Transgenic Core Facility).

Animal experiments

After initial backcrossing to C57BL/6, F1 offspring of a DK5.25 chimera (Figure 3E) were bred to $Rosa26^{FLPo}$ deletor mice [44], obtained from the Jackson Laboratory (Bar Harbor, ME), to delete the FRT-flanked neo^R cassette. neo^R excision yielded the $Muc1^{IRES\text{-}CreERT2}$ ($Muc1^{IC2}$) allele (data not shown). Multiplex PCR genotyping, producing bands of 357 bp (wildtype) and 464 bp (mutant), was performed using oligos: wt forward: 5'-AATGGCAGTAGCAGTCTCTC-3'; wt reverse: 5'-CACAGCTGGCATAACTAACA-3'; and mutant reverse: 5'-CCACAACTATCCAACTCACA-3'. $Muc1^{IC2}$ mice were maintained on a CD1 outbred background. Cre reporter mice $Rosa26^{EYFP}$ [25] and $Rosa26^{LacZ}$ [26] were obtained from the Jackson Laboratory.

Tamoxifen (Sigma T-5648) was dissolved in corn oil, and administered by oral gavage at doses of 5-10 mg to adult mice, or 2 mg by subcutaneous injection of neonates. For timed-pregnancy studies, noon on the day after vaginal plugging was considered embryonic day 0.5 (E0.5). All animal procedures were approved by the Institutional Animal Care and Use Committee.

Immunostaining and analysis

Tissue fixation, processing and immunostaining were performed essentially as described [45]. Tissues were fixed with 4% paraformaldehyde (PFA) in PBS for 1-2 hrs at 4°C, embedded in OCT and cryosectioned at 7-8 μm thickness. Primary antibodies used in this study are listed in Table 3. Secondary antibodies were purchased from Jackson Immunoresearch. To calculate labeling efficiencies, we photographed 5-12 randomly selected 20× fields per stained specimen, across 4-8 sections separated by 100-150 μm. The total number of each cell type (DAPI for total cells per field, LacZ or GFP for $Muc1^{IC2}$-labeled cells, insulin and glucagon for endocrine cells, amylase, cytokeratin-19 and DBA lectin for exocrine cells) was determined using the Analyze Particles function of ImageJ (NIH). Double-positive cells were detected by additive image overlay, in ImageJ, of the DAPI channel with lineage$^+$ and marker$^+$ staining. Accuracy of counts was confirmed by eye in Adobe Photoshop for random samples. Calculations and graphs were generated with Microsoft Excel and R http://www.r-project.org.

Acinar isolation and staining

Acini were isolated by sequential trypsin and collagenase P digestion of minced dorsal pancreas, as described [46], PFA-fixed for 15 min and adhered to microscope slides by cytospin (Thermo-Fisher). Cytospin slides were

Table 3 Primary antibodies used in this study

Antigen	Species	Source	Catalog #	Dilution
amylase	sheep	BioGenesis	0480-0104	1:2500
amylase	rabbit	Sigma	A8273	1:1000
cytokeratin-19	rat	Developmental Studies Hybridoma Bank	TROMA-3	1:50
cytokeratin-19	rabbit	Ben Stanger (University of Pennsylvania)		1:1000
C-peptide	rabbit	Linco	4020-01	1:2500
C-peptide	goat	Linco	4023-01	1:5000
E-cadherin	rat	Zymed/Invitrogen	13-1900	1:2000
GFP	rabbit	Abcam	ab290	1:4000
GFP	goat	Rockland	600-101-215	1:2500
glucagon	rabbit	Zymed/Invitrogen	18-0064	1:250
glucagon	guinea pig	Linco	4031-01F	1:2500
LacZ	rabbit	Cappel/MP	55976	1:2000
Muc1	hamster	NeoMarkers	HM-1630-P1	1:500
Neurog3	mouse	Developmental Studies Hybridoma Bank	I25A1B3	1:75

stained as per cryosections. For wholemount immunofluorescence of intact tissue, small pieces of the dorsal pancreas were excised and PFA-fixed as above, washed with PBS, permeabilized with 1% Triton X-100 in PBS and stained with primary and secondary antibodies (overnight incubations, followed by extensive PBS/0.1% Tween-20 washes).

Abbreviations

Cpa1: carboxypeptidase A1; CK19: cytokeratin-19; DAPI: 4',6-diamidino-2-phenylindole; DBA: Dolichos biflorus agglutinin; EYFP: enhanced yellow fluorescent protein; FIAU: 1-(-2-deoxy-2-fluoro-1-β-D-arabinofuranosyl)-5-iodouracil; GFP: green fluorescent protein; IRES: internal ribosome entry sequence; PFA: paraformaldehyde; TM: tamoxifen.

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Authors' contributions

LCM and DK developed the study concept and design. LCM provided input on methodology and analysis, and supervised the study. DK performed experiments, acquired and analyzed data. DK and LCM interpreted the data and wrote the manuscript. Both authors have read and approved the final manuscript.

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CHAPTER 4

MUC1^{+} EXOCRINE CELLS DO NOT CONTRIBUTE TO NEW β-CELLS AFTER PANCREATIC DUCT LIGATION

Introduction

Autoimmune destruction of insulin-producing beta (β)-cells from the pancreas results in type 1 diabetes. Although lost β -cells in this disease can be replaced by islet transplantation, donor organs are scarce. It is therefore widely hoped that adult exocrine tissue could serve as a new source for transplantable β-cells. Indeed, this possibility has been aggressively pursued, through culture-based studies, for at least a decade (Baeyens et al., 2005; Bonner-Weir et al., 2000; Gao et al., 2005; Hao et al., 2006; Ramiya et al., 2000; Yatoh et al., 2007). These studies remain, however, largely inconclusive, since they are not supported by *in vivo* studies in the mouse, which indicate that adult β-cells are maintained by self-renewal rather than neogenesis (Brennand et al., 2007; Dor et al., 2004; Nir et al., 2007; Teta et al., 2007). On the other hand, experiments *in vitro* and injury models *in vivo* may provide as yet-unidentified signals sufficient to trigger neogenesis.

Before discussing the possibility of injury-induced neogenesis, we will briefly consider the more settled question of where embryonic β-cells come from. Descriptive studies suggested that islet cells arise from duct-like structures (Slack, 1995), and

Neurogenin-3 (*Neurog3*), a transcriptional "master regulator" of endocrine development, is expressed by duct-localized, endocrine-restricted precursor cells (Gradwohl et al., 2000; Gu et al., 2002; Johansson et al., 2007; Schonhoff et al., 2004; Schwitzgebel et al., 2000). This hypothesis was only recently proven to be correct through the development of novel duct-specific Cre lines allowing for lineage tracing of the embryonic ducts. We and others demonstrated that embryonic islets indeed arise from *Neurog3*$^{+}$ cells that reside and originate from within the ductal epithelium (Furuyama et al., 2011; Kopinke and Murtaugh, 2010; Kopp et al., 2011; Solar et al., 2009). β-cell expansion and homeostasis in the adult, however, appear to be driven by proliferation of already-differentiated β-cells (Bouwens and Rooman, 2005; Dhawan et al., 2007). This is supported by lineage-tracing studies, using transgenic mice in which existing β-cells can be marked and followed (Brennand et al., 2007; Dor et al., 2004; Nir et al., 2007). Although these studies indicate that nearly all adult β-cells arise from pre-existing β-cells, even in certain injury contexts, they might miss low levels of neogenesis, below the level of sensitivity imposed by experimental error (Murtaugh and Kopinke, 2008). A more robust approach would be to label exclusively exocrine ducts and acini prior to injury, and then determine if any labeled cells contribute to new β-cells -- in such a paradigm, even a very small number of neogenic cells would be detectable.

More recently, compelling evidence has been provided to suggest the existence of β-cell neogenesis following injury induced by pancreatic duct ligation (PDL) (Wang et al., 1995; Xu et al., 2008). The key features of this model are: (i) duct cells rapidly expand, while acini undergo apoptosis; (ii) the ligated (splenic) lobe experiences a rapid and sustained doubling of β-cell mass, while the rest of the organ is unaffected; (iii) this

increase in β-cell mass is accompanied by, and dependent on, reactivation of *Neurog3* expression in the ducts. Nonetheless, definitive proof for a ductal origin of new β-cells post-PDL is still awaiting confirmation, since the necessary tools to perform lineage tracing of ducts have only recently been developed.

In this study, we aimed to address the open question of whether new β-cells can originate from the ductal epithelium following duct ligation injury. After establishing this injury model in our laboratory, we combined our tamoxifen-inducible $Muc1^{IC2}$ line with Cre-dependent reporter mice to perform rigorous lineage tracing of exocrine cells during PDL. Our findings suggest that the source for new β-cells is independent of the $Muc1^{+}$ exocrine population. It further indicates that the origin must either be a Muc1-negative duct cell or that neogenesis does not occur after PDL.

Materials and methods

Mice

$Muc1^{IC2}$ (Kopinke and Murtaugh, 2010), $R26R^{EYFP}$ (Srinivas et al., 2001), $R26R^{LacZ}$ (Soriano, 1999) and $Neurog3^{EGFP}$ (Lee et al., 2002) mice have been described previously. Tamoxifen (TM) was dissolved in corn oil and administered by oral gavage at doses of 10 mg. All experiments were carried out according to institutional guidelines. Pancreatic duct ligations were performed as described (Kopinke et al., 2011). In brief, after anaesthetization with isoflurane the abdomen was shaved and cleaned with iodine. After Bupivacaine (0.25%) injections along the incision site, we accessed the peritoneum via a horizontal incision in the upper-left abdomen, deflected the liver and isolated the stomach with a hemostat, gently exposed and immobilized the splenic pancreas with

forceps and cotton swab, and tied off the splenic lobe with a monofilament (5-0) suture, sparing the splenic vessels. In sham-operated mice, we similarly exposed the pancreas, but returned it to the body cavity without ligation; these pancreata appeared completely normal (data not shown). Buprenorphine (0.05 mg/kg) was administered subcutaneously every 12 hours for the first 72 hours postsurgery. All animal procedures were approved by the Institutional Animal Care and Use Committee of the University of Utah.

Tissue processing and histology

Immunostaining and analysis were performed as previously described (Kopinke et al., 2011; Kopinke and Murtaugh, 2010). The following primary antibodies were used: rat anti-cytokeratin-19 1:50 (Developmental Studies Hybridoma Bank), rat anti-E-cadherin 1:2000 (Zymed), rabbit anti-GFP 1:4000 (Abcam), goat anti-GFP 1:2500 (Rockland), guinea pig anti-glucagon 1:2500 (Linco), rabbit anti-glucagon 1:2500 (Zymed), guinea pig anti-Insulin 1:2000 (Dako), rabbit anti-LacZ 1:2000 (Cappel) and guinea pig anti-Pdx1 1:10000 (Chris Wright, Vanderbilt University). All secondary antibodies (raised in donkey) were obtained from Jackson Immunoresearch. For quantifications, co-immunofluorescence was determined using the Analyze Particles function of ImageJ (NIH) and confirmed by eye in Adobe Photoshop as previously described (Kopinke et al., 2011; Kopinke and Murtaugh, 2010).

Results

Successful recapitulation of pancreatic duct ligation injury

Since we did not observe any contribution to new beta cells from Muc1-expressing ducts during postnatal expansion or adult homeostasis (Kopinke and Murtaugh, 2010), we wanted to test the differentiation potential of ductal and acinar cells during PDL, an injury model suggested to induce β-cell neogenesis (Wang et al., 1995; Xu et al., 2008).

Our initial goal was to faithfully reproduce the previously reported phenotype (Wang et al., 1995; Xu et al., 2008), which we achieved with the help of Dr. Courtney Scaife (Department of Surgery). Using CD1 wild type mice, we defined PDL as successful when the portion distal to the ligation turned translucent, acinar tissue was lost and replaced by inflammatory and epithelial ductal-like cells, and more islets were present per ligated area compared to the control portion 7 days post-PDL (Fig. 4.1). Our success rate was in the range of 40-50% and seemed inversely correlated with body weight, such that surgeries usually work in young and lean mice and almost always fail in older and heavier (>35 g) mice.

Another key feature of PDL is re-expression of *Neurog3*$^{+}$ cells within duct-like cells of the ligated portion. We have attempted without success to detect such cells in our experimental specimens, using three different antibodies that readily detect Neurog3-expressing cells in the embryonic pancreas. It is possible that the levels of Neurog3 expression are below the sensitivity threshold of our antibodies. Interestingly, a recent study indicates that Neurog3 is expressed in differentiated islet cells (Wang et al., 2009), contradicting earlier studies in which Neurog3 expression could not be detected in

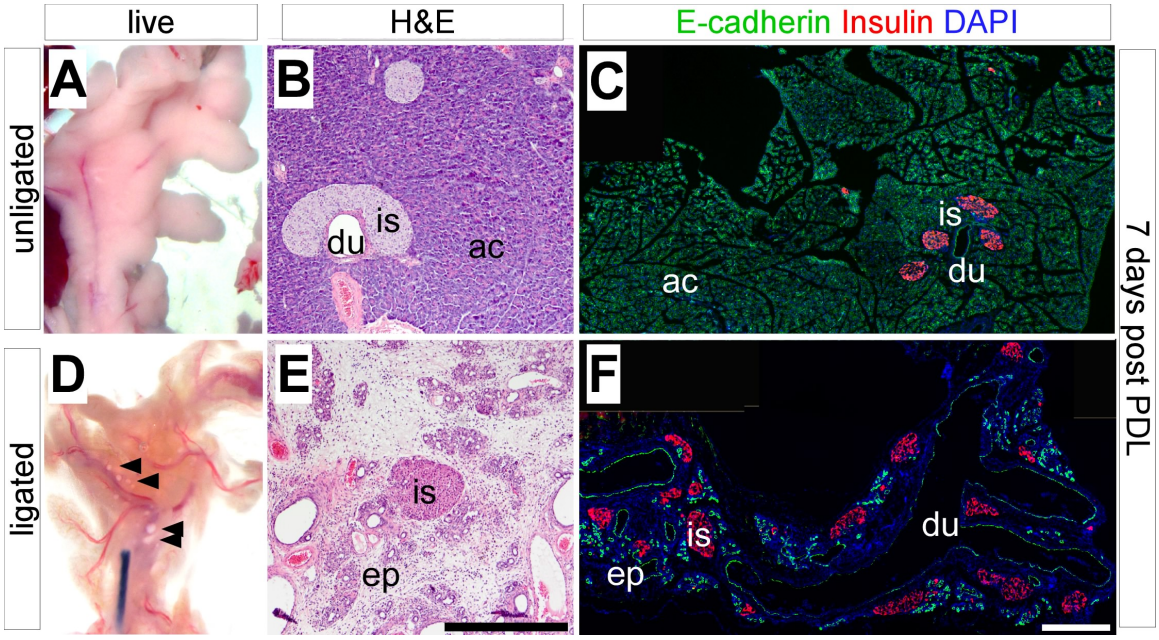

Figure 4.1. Pancreatic duct ligation. Morphological characterization of unligated (A-C) and ligated pancreatic lobes (D-F) from 8-week-old CD1 wild-type mice, 7 days after PDL injury. **(A-B)** Control pancreata are of white color (A) and exhibit normal architecture with acinar cells (ac), islets (is) and ducts (du) by hematoxylin and eosin staining (B). **(D-E)** After ligation, pancreatic tissue appears yellow and more translucent, such that individual islets become visible (D, arrowheads). In addition, the ligated lobes have undergone complete replacement of acinar tissue by ductal-like epithelial structures (ep) with interspersed islets (E). C and F are fields from a single section through an operated pancreas, capturing both the unligated (C) and ligated area (F). E-cadherin (green) marks acinar and ductal tissue in the unligated pancreas, as well as the residual epithelial and ductal structures remaining after PDL. The area occupied by insulin-expressing islets (red) appear to increase after PDL. Scale bars: 500 μm.

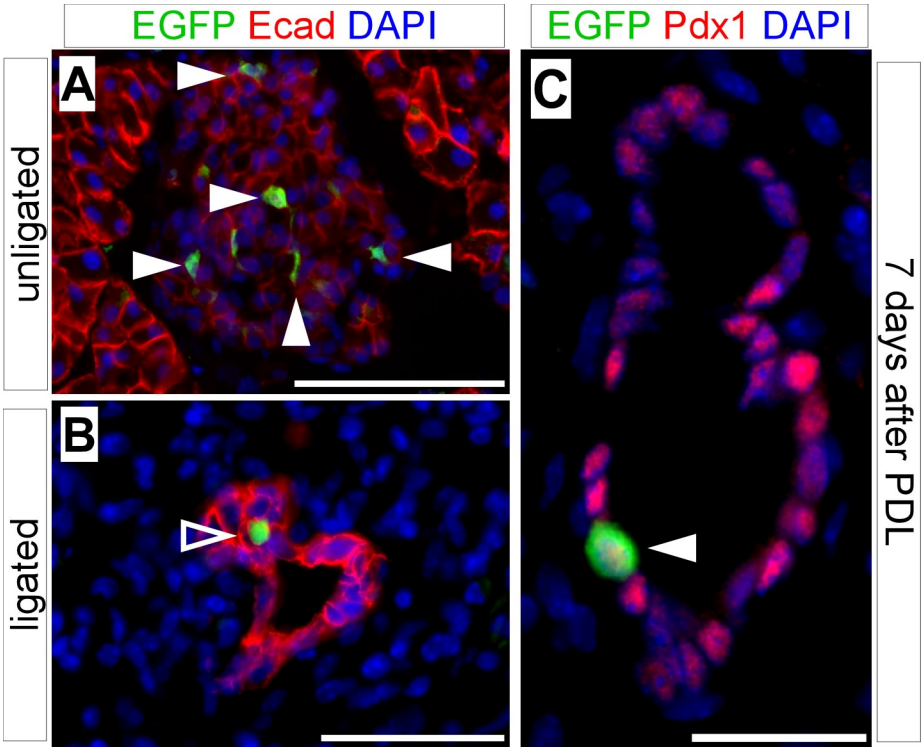

Figure 4.2. Re-expression of embryonic progenitor markers. PDL was performed on 8-week-old *Neurog3*$^{EGFP/+}$ reporter mice, and EGFP expression (green) was analyzed 7 days after injury. **(A-B)** While weak *Neurog3* expression can be detected only in islet cells (arrowheads) in the unligated portion, rare GFP^{+} cells can be found also in E-cadherin^{+} (red) epithelial clusters (open arrowhead) after ligation. **(C)** PDL injury also causes re-expression of the progenitor marker Pdx1 (red) within duct-like structures as well as within *Neurog3*$^{+}$ cells (green, arrowhead). Scale bars: A, 100 μm; B, 50 μm; C, 25 μm.

hormone-positive cells (Gradwohl et al., 2000; Schwitzgebel et al., 2000). In this study, detection of Neurog3 in adult islets was achieved using a *Neurog3*^{EGFP} knock-in mouse in which GFP expression acts as a surrogate for Neurog3 activity (Lee et al., 2002). To enhance our ability to detect rare or faintly-expressing *Neurog3*$^{+}$ cells, we obtained *Neurog3*^{EGFP} mice and performed PDL experiments using this strain (n=5). As described by others (Wang et al., 2009), we could detect Neurog3 expression in islet cells within the unligated control area (Fig. 4.2A). *Neurog3*$^{+}$ cells could also be found in epithelial duct-like cells within the ligated portion, but at a very low frequency (Fig. 4.2B). Duct ligation injury also caused more widespread up-regulation of another early progenitor marker, Pdx1, within the duct-like cells (Fig. 4.2C).

These results demonstrate that our surgery technique is successful in recapitulating known hallmarks of PDL injury. During pancreas development, all progenitors express Pdx1 while new islet cells originate from *Neurog3*$^{+}$ cells of the ductal epithelium (Gu et al., 2002; Kopinke and Murtaugh, 2010; Kopp et al., 2011; Solar et al., 2009). The re-expression of these early progenitor markers within duct-like cells after PDL suggests that this injury might cause a recapitulation of embryonic developmental mechanisms within the adult duct, providing a potential basis for induction of β-cell neogenesis in this model.

Muc1^{+} exocrine cells do not contribute to new β-cells during injury.

After establishing the PDL injury model, we wanted to determine whether duct cells could act as endocrine precursors in this setting. To perform lineage tracing of

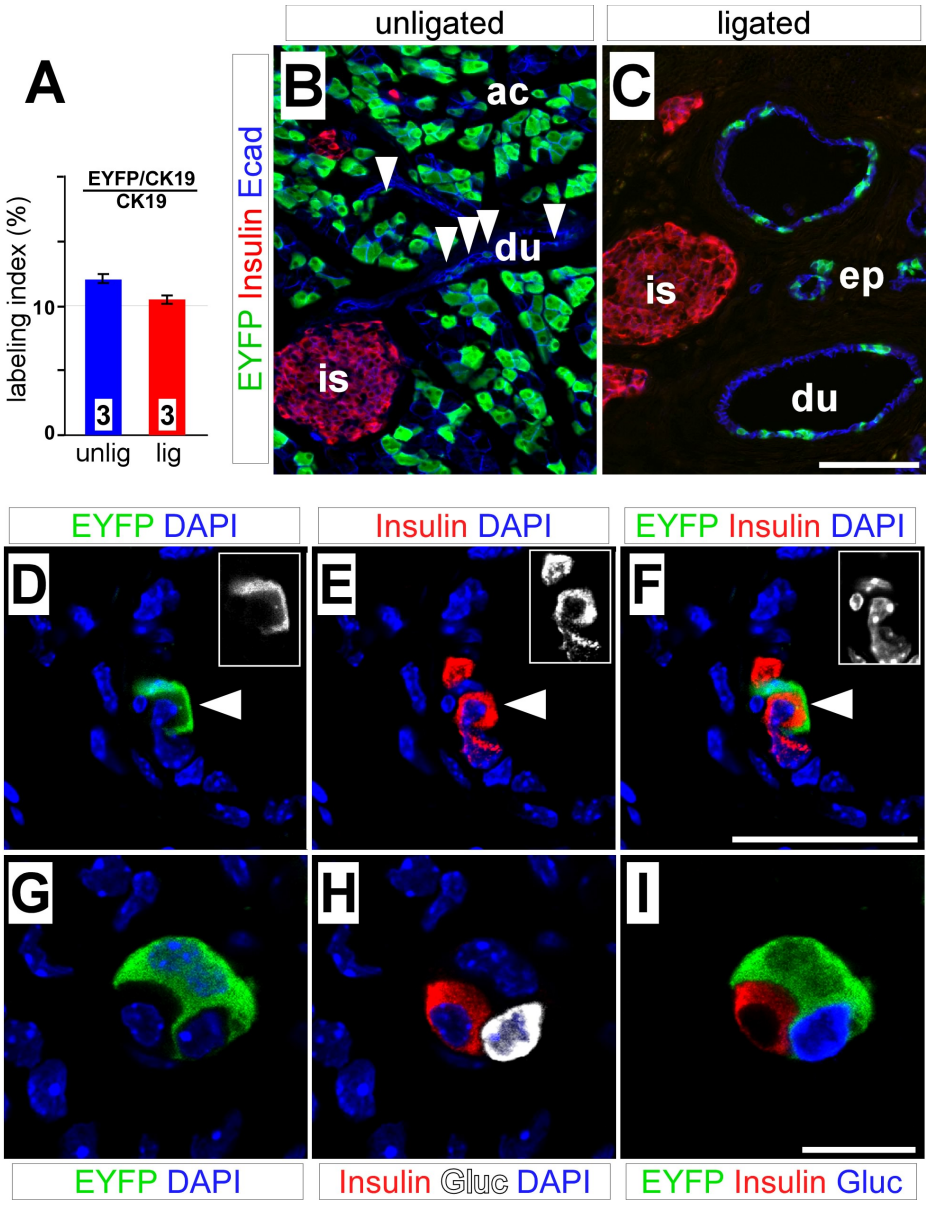

Figure 4.3. PDL does not cause *Muc1*$^{+}$ exocrine cells to undergo neogenesis. Recombination was induced in 2-month-old *Muc1*$^{IC2/+}$; *R26R*$^{EYFP/+}$ mice, one month prior to PDL and the EYFP lineage label (green) analyzed 7 days after injury. **(A)** Quantification of EYFP duct labeling in ligated and unligated areas. Number of mice analyzed is indicated in each bar. Data are represented as mean +/- SEM. **(B-C)** While *Muc1*^{IC2} robustly marks acinar cells (ac) and ducts (du) in the unligated (A) as well as epithelial duct-like structures (ep) in the ligated portion (B), the lineage label was never detected in insulin-expressing β-cells (red) of the healthy or injured portion (A-B). **(D-I)** Confocal microscopy of EYFP^{+} and endocrine cells within ducts (D-F) or small clusters (G-I), 7 days post-PDL. EYFP-labeled cells can be found in close contact with either insulin^{+} cells (D-F, arrowhead) or insulin^{+} and glucagon^{+} α-cells (G-I) but EYFP is never expressed by endocrine cells. Scale bars: A-B, 100 μm; D-F, 50 μm; G-I, 25 μm.

ducts, we utilized our recently generated TM-inducible *Muc1*^{IC2} Cre-line (Kopinke and Murtaugh, 2010). Muc1 is expressed by duct and acinar cells but, since acinar cells do not contribute to new islets during normal homeostasis and injury (Desai et al., 2007; Means et al., 2005; Zhou et al., 2007), we could treat the labeling of acinar cells as "background". *Muc1*$^{IC2/+}$*; R26R*$^{EYFP/+}$mice, which carry a Cre-dependent EYFP reporter allele (Srinivas et al., 2001), received three doses of 10 mg TM 30 days before surgery, after which they were chased for an additional 7 days. This pulse-chase scheme was chosen to maximize labeling efficiency of exocrine cells and also to allow for clearance of TM from the bloodstream before the injury.

We have obtained successful PDLs with a total of 16 tamoxifen-treated *Muc1*$^{IC2/+}$*; R26R*$^{EYFP/+}$ mice. We observed ~11 % EYFP labeling within the cytokeratin-19 (CK19)-expressing ductal epithelium of the ligated pancreata, and 12% in the unligated portion (Fig. 4.3A). Interestingly, *Muc1*^{IC2} consistently labels a 2-4-fold greater proportion of acinar cells than ducts in the unligated portions of our PDL specimens (Fig. 4.3B). The fact that the labeling indices of ducts are almost the same between unligated and ligated pancreata further suggests that the latter population does not derive from the more highly-labeled acinar population. Although acinar cells can convert to a duct-like phenotype *in vivo* (Blaine et al., 2010), our results suggest that this does not occur after PDL, and that acini are simply destroyed in this model. Surprisingly, we did not observe any labeling of β-cells in the 12 *Muc1*$^{IC2/+}$*; R26R*$^{EYFP/+}$ PDL specimens analyzed. In fact, after inspecting ~10,000 β-cells present per ligated pancreas, not a single EYFP^{+} β-cell was found (Fig. 4.3 and data not shown). Confocal analysis indicated that although small clusters of β- and α-cells could be found juxtaposed

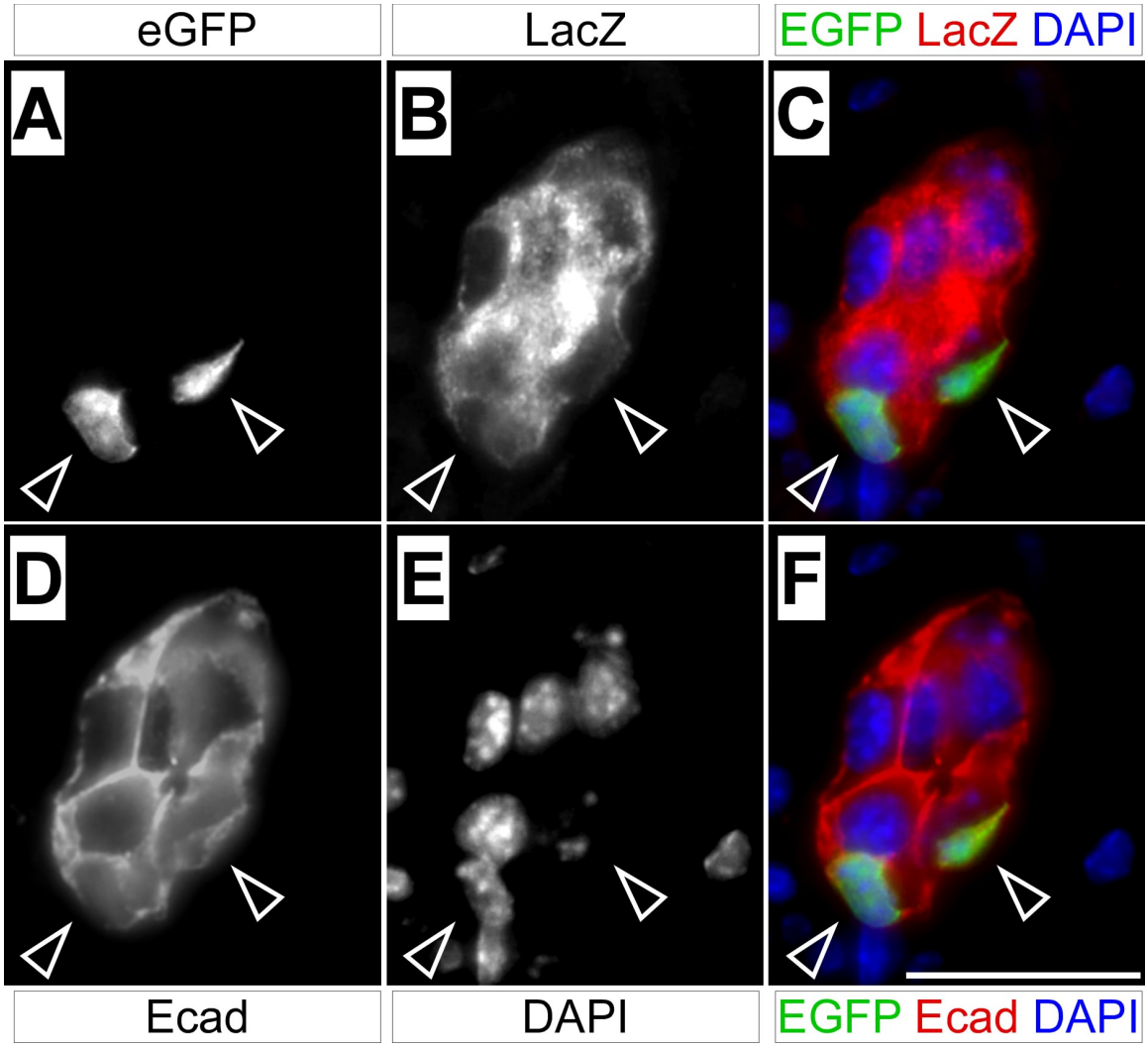

Figure 4.4. Neurog3 is expressed within Muc1-derived ductal structures. *Muc1*$^{IC2/+}$; *R26R*$^{LacZ/+}$; *Neurog3*$^{eGFP/+}$ mice received 3x10 mg TM one month prior to ligation, and were analyzed for the LacZ lineage label (red) and EGFP expression (green) 7 days after surgery. **(A-F)** Two Neurog3-expressing cells are present within a small cluster of Muc1-derived cells and carry the lineage label (arrowheads). This indicates that, despite the fact that *Neurog3* is expressed within Muc1 lineage-derived ducts, these cells do not adopt a β-cell fate after injury. Scale bar: A-F, 50 μm.

with EYFP^{+} cells, they were never labeled (Fig. 4.3D-I). Thus, our findings suggest that if neogenesis does occur after PDL, the source is independent of the exocrine population labeled by *Muc1*^{IC2}. We cannot, however, exclude the possibility that a Muc1-negative duct population might represent a source for neogenesis, although we have previously shown that Muc1 is expressed throughout the ductal network (Kopinke and Murtaugh, 2010).

By performing lineage tracing of *Muc1*$^{+}$ cells in combination with the *Neurog3*^{EGFP} reporter, we sought to determine whether the *Muc1*$^{+}$ population is at least capable of inducing Neurog3 expression. Since this reporter expresses GFP, we utilized the *R26R*^{LacZ} reporter instead of EYFP for lineage tracing of *Muc1*$^{+}$ exocrine cells. We used the same pulse-chase strategy as before to induce recombination in *Muc1*$^{IC2/+}$; *R26R*$^{LacZP/+}$; *Neurog3*$^{EGFP/+}$ mice one month before PDL (n=6). The distribution of the LacZ lineage label was indistinguishable from the EYFP expression seen before, demonstrating the interchangeability of the reporter alleles (data not shown). Interestingly, we were able to detect a few examples of Muc1-derived cells expressing *Neurog3* (Fig. 4.4). Although these are very rare, our ability to detect them confirms that *Neurog3* can be expressed by Muc1 lineage-labeled duct cells. Since we never detected the lineage label in islet cells, our finding suggests that *Neurog3*$^{+}$ Muc1-derived ducts do not adopt a β-cell fate.

Discussion

A recent study suggested that new β-cells originate from the ductal compartment following PDL, but lacked proof from lineage tracing (Xu et al., 2008). Our experiments

use this approach, the most stringent test for changes in cellular phenotype, to assess the differentiation capacity of adult duct and acinar cells in the PDL injury model. Our findings suggest, however, that adult *Muc1*$^{+}$ ducts, unlike their counterparts in the embryo, have no ability to give rise to new β-cells.

Since 2008, nine different TM-inducible CreERT have been described to label, to varying degree, the ductal compartment and five of those have been used to perform lineage tracing after PDL (see Chapter 5 for a more detailed discussion). Our findings arguing against neogenesis are identical to those obtained with *Hnf1bCreER* (Solar et al., 2009), *Hes1*^{C2} (Kopinke et al., 2011), *Sox9CreER* (Kopp et al., 2011) and *Sox9*$^{IRES\text{-}CreERT}$ (Furuyama et al., 2011). Taken together, these studies strongly suggest that new β-cells arise after PDL either from pre-existing β-cells, or from a duct subpopulation that expresses neither *Muc1*, *Hnf1b, Hes1* nor *Sox9* (Inada et al., 2008).

If neogenesis is not induced by PDL, what potential role might Neurog3 play in this injury model? It was recently shown that Neurog3 is expressed in mature islets using three independent lines of *Neurog3* knock-in reporter mice and mRNA/protein-based assays. By inactivating *Neurog3* in insulin-expressing β-cells, it was further shown that Neurog3 is required for β-cell maturation and maintenance during postnatal expansion and homeostasis (Wang et al., 2009). *Neurog3* knockdown using shRNA during PDL also inhibits the expansion and proliferation of islet cells normally seen after injury (Xu et al., 2008). These results not only highlight the important role of adult Neurog3 expression but could also provide an alternative explanation for the shRNA knockdown results, which might have disrupted expansion of pre-existing β-cells rather than neogenesis.

Additionally, the finding that PDL induces doubling of β-cell mass was recently put into question. Traditionally, total β-cell mass was determined by measuring the area occupied by insulin staining within several representative sections, divided by the total section area and multiplied by the weight of the pancreas (Solar et al., 2009; Wang et al., 1995; Xu et al., 2008). This so-called morphometric measurement of β-cell mass is, however, skewed by the total loss of the acinar tissue, which represent ~90% of the pancreas, and the concomitant edema (Fig. 4.1C and F). As an alternative approach, Kopp et al. measured whole pancreas insulin content and found that there was no change between unligated and ligated pancreata, raising the possibility that the β-cell compartment does not expand in response to PDL (Kopp et al., 2011).

Generation of new β-cells from endogenous precursors could provide an attractive and noninvasive method to treat human diabetes; our work suggests that if such precursors exist, they are not located within the ductal compartment. Our findings not only add to the growing body of evidence against β-cell neogenesis but also raise doubts on the suitability of the PDL model as a starting point for further investigations aimed to increase β-cell mass.

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CHAPTER 5

LINEAGE TRACING REVEALS THE DYNAMIC CONTRIBUTION OF *HES1*$^{+}$ CELLS TO THE DEVELOPING AND ADULT PANCREAS

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Lineage tracing reveals the dynamic contribution of *Hes1*$^{+}$ cells to the developing and adult pancreas

Daniel Kopinke[1], Marisa Brailsford[1], Jill E. Shea[2], Rebecca Leavitt[1], Courtney L. Scaife[2] and L. Charles Murtaugh[1,*]

SUMMARY

Notch signaling regulates numerous developmental processes, often acting either to promote one cell fate over another or else to inhibit differentiation altogether. In the embryonic pancreas, Notch and its target gene *Hes1* are thought to inhibit endocrine and exocrine specification. Although differentiated cells appear to downregulate *Hes1*, it is unknown whether *Hes1* expression marks multipotent progenitors, or else lineage-restricted precursors. Moreover, although rare cells of the adult pancreas express *Hes1*, it is unknown whether these represent a specialized progenitor-like population. To address these issues, we developed a mouse *Hes1*^{CreERT2} knock-in allele to inducibly mark *Hes1*$^{+}$ cells and their descendants. We find that *Hes1* expression in the early embryonic pancreas identifies multipotent, Notch-responsive progenitors, differentiation of which is blocked by activated Notch. In later embryogenesis, *Hes1* marks exocrine-restricted progenitors, in which activated Notch promotes ductal differentiation. In the adult pancreas, *Hes1* expression persists in rare differentiated cells, particularly terminal duct or centroacinar cells. Although we find that *Hes1*$^{+}$ cells in the resting or injured pancreas do not behave as adult stem cells for insulin-producing beta (β)-cells, *Hes1* expression does identify stem cells throughout the small and large intestine. Together, these studies clarify the roles of Notch and *Hes1* in the developing and adult pancreas, and open new avenues to study Notch signaling in this and other tissues.

KEY WORDS: Pancreas, Hes1, Notch, Stem cell, Mouse

INTRODUCTION

The vertebrate pancreas comprises three major cell types: endocrine islets, which include insulin-producing β-cells; and a network of exocrine acinar and duct cells, which are responsible for producing and transporting digestive enzymes, respectively. The Notch signaling pathway has been implicated in several aspects of pancreatic cell fate determination, beginning with the finding that mouse embryos lacking various Notch components, including the downstream target gene *Hes1*, exhibit overproduction of endocrine cells (Apelqvist et al., 1999; Jensen et al., 2000). Hes1 can repress the promoter of *Neurog3*, a crucial pro-endocrine transcription factor, and de-repression of *Neurog3* in the absence of *Hes1* may drive excessive endocrine differentiation (Apelqvist et al., 1999; Jensen et al., 2000; Lee et al., 2001). In gain-of-function experiments, Notch also inhibits exocrine acinar cell development, promoting instead progenitor maintenance (Esni et al., 2004; Hald et al., 2003; Murtaugh et al., 2003). These findings are corroborated by studies in zebrafish (Esni et al., 2004; Yee et al., 2005; Zecchin et al., 2006), and conform to a generic conception of Notch as regulating cell fate throughout animal development (Lai, 2004).

The Notch pathway knockout phenotypes implied that the early pancreas comprised multipotent cells, the differentiation of which was held in check by Notch signaling (Apelqvist et al., 1999; Jensen et al., 2000). Lineage-tracing studies suggest that multipotent progenitors reside in the 'tips' of the embryonic pancreatic epithelium, the expansion of which leaves behind 'trunks' that give rise to ducts and islets (Kopinke and Murtaugh, 2010; Solar et al., 2009; Zhou et al., 2007). How Notch regulates this process is unknown, although it may signal through *Hes1* to repress *Neurog3* (Lee et al., 2001) and control the balance of duct and islet differentiation. Contradicting this model, however, deletion of *Notch1* and *Notch2*, the major receptors expressed in the pancreas, has little effect on late embryonic islet development (Nakhai et al., 2008).

Whether progenitor cells persist in the adult pancreas, particularly for insulin-producing β-cells, remains controversial. Two lineage tracing approaches have been taken to address this issue: 'pulse-chase' labeling of mature islet cells, to detect changes in labeling frequency caused by differentiation of new β-cells (neogenesis); or marking acini and/or ducts, to determine whether they can contribute to β-cells. The former studies argue against β-cell neogenesis (Dor et al., 2004), and the latter indicate that neogenesis is either non-existent (Desai et al., 2007; Kopinke and Murtaugh, 2010; Solar et al., 2009) or rare (Inada et al., 2008) in the uninjured pancreas. Pancreatic injury, in particular caused by ligation of the main duct, has been proposed to induce facultative neogenesis from acinar or duct cells (Wang et al., 1995; Xu et al., 2008), for which exist both contradictory and supporting lineage tracing data (Inada et al., 2008; Solar et al., 2009). As previous approaches used mature duct or acinar marker genes to drive Cre expression, however, they may have excluded specialized adult progenitor cells. These might include centroacinar cells, terminal elements of the exocrine ductal network in which Notch-*Hes1* signaling appears particularly high

[1]Department of Human Genetics, University of Utah School of Medicine, Salt Lake City, UT 84112, USA. [2]Department of Surgery, University of Utah School of Medicine, Salt Lake City, UT 84132, USA.

*Author for correspondence (murtaugh@genetics.utah.edu)

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(Miyamoto et al., 2003; Parsons et al., 2009; Stanger et al., 2005). These cells have been suggested to generate new β-cells following injury (Hayashi et al., 2003; Nagasao et al., 2003), and they can give rise to both acinar and islet cells following isolation and culture (Rovira et al., 2010).

To understand how and when Notch-*Hes1* signaling regulates pancreatic progenitor cells, we generated 'knock-in' mice in which the tamoxifen-dependent CreERT2 recombinase is targeted to the *Hes1* locus. With these mice, we have analyzed the stage-specific differentiation potential of Notch-responsive cells in the embryonic pancreas, revealing a novel shift from multipotent to exocrine-restricted progenitor cells. This parallels a shift in the cellular response to Notch, from arresting differentiation to promoting duct cell specification. In the adult, we find that *Hes1*$^{+}$ duct and centroacinar cells appear to be fixed in their fate, and do not detectably contribute to β-cells, even after duct ligation injury. Ours is the first study to address the fate of Notch-responsive cells in any adult tissue, and supports an emerging model that lineage boundaries in the pancreas are normally fixed at birth.

MATERIALS AND METHODS

Mice

We used bacterial recombineering (Liu et al., 2003) to generate a *Hes1*$^{CreERT2\text{-}neoR}$ targeting vector, in which most of the *Hes1* open reading frame, including the bHLH domain, is replaced by that of *CreERT2* (Feil et al., 1997), linked to an FRT-flanked *neo*^{R} cassette (see Fig. S1A in the supplementary material). This was electroporated into R1 ES cells (Nagy et al., 1993), generously provided by Mario Capecchi (University of Utah, USA), and G418-resistant ES cell clones were screened by Southern blotting and PCR (see Fig. S1B in the supplementary material and data not shown). Germline chimeras were derived by the University of Utah Transgenic Core Facility. The *neo*^{R} cassette was excised in vivo by breeding to *Rosa26*^{FLPe} (Farley et al., 2000), obtained from the Jackson Laboratory. Cre reporter mice *R26R*^{EYFP} (Srinivas et al., 2001) and *R26R*^{LacZ} (Soriano, 1999) were obtained from the Jackson Laboratory. *Rosa26*^{NIC} (Murtaugh et al., 2003) and *Pdx1Cre* mice (Gu et al., 2002) were provided by Doug Melton (Harvard University, MA, USA). *Ctnnb1*$^{lox(ex3)}$ mice (Harada et al., 1999) were provided by Makoto Mark Taketo (Kyoto University, Japan). Tamoxifen (Sigma T-5648) was dissolved in corn oil and administered by oral gavage.

Explant cultures were established from E11.5 dorsal pancreatic buds, with embryos genotyped by PCR immediately after dissection. Buds were cultured at the air-medium interface, on Millicell-CM filters (0.4 μm pore size; Millipore), in DMEM supplemented with 10% FBS and antibiotics. As indicated, explants were treated with the γ-secretase inhibitor DAPT (10 μM final; Calbiochem) to inhibit Notch signaling (Dovey et al., 2001), or with vehicle alone as a control (0.1% DMSO final), 24 hours prior to treatment with 250 nM 4-hydroxytamoxifen to activate CreERT2.

Pancreatic duct ligations were performed as described previously (Scoggins et al., 2000; Solar et al., 2009; Xu et al., 2008), following a protocol approved by the University of Utah IACUC. Briefly, following midline laparotomy, the stomach and ascending colon were deflected to access the dorsal pancreas. The dorsal pancreas was lifted and stretched slightly with a surgical probe, and a monofilament ligature placed near the base, sparing the splenic vessels. Surgery was performed under isoflurane anesthesia, and mice received an analgesic dose of buprenorphine (50 μg/kg body weight) immediately after surgery.

Immunostaining and lineage analysis

Tissues were fixed and immunostained essentially as described previously (Kopinke and Murtaugh, 2010). Immunofluorescence was performed on frozen sections (7-8 μm) of tissue fixed in 4% paraformaldehyde/PBS (4°C, 1-2 hours). Other analyses used paraffin sections (6 μm) of tissue fixed in zinc-buffered formalin (room temperature, overnight). Primary antibodies used in this study are listed in Table 1; where indicated, sections were also stained with Dolichos biflorus agglutinin (DBA) lectin (Vector Laboratories), which marks duct cells (Kobayashi et al., 2002). Staining was analyzed by compound fluorescent or light microscopy, using MicroSuite software (Olympus). Photomicrographic images were processed using Adobe Photoshop, with parallel images processed identically.

Our lineage analysis approach, following that of others (Gu et al., 2002; Solar et al., 2009; Zhou et al., 2007), is schematized in Fig. S2 in the supplementary material. One or two randomly chosen fields, comprising 800-1000 DAPI^{+} cells each, were photographed from each of four to ten widely separated sections. For studies of E17.5 embryonic pancreata, we collected six to eight sections per slide, separated by 80-100 μm and spanning the entire pancreas as well as adjacent stomach and duodenum. For uninjured adult pancreata, we embedded and sectioned one half of each dorsal pancreas, collecting four or five sections per slide spaced at least 100 μm apart. For duct ligations, we sectioned the entire dorsal pancreas (ligated and unligated regions), collecting eight to ten sections per slide separated by at least 120 μm. Total numbers of mice, fields and cells scored in each experiment are listed in Tables S2 and S3 in the supplementary material.

Labeling efficiencies were derived from co-immunofluorescence, using the Analyze Particles function of ImageJ (NIH) (Kopinke and Murtaugh, 2010). Counting accuracy was confirmed by eye in Adobe Photoshop for random samples. To exclude the possibility that cells were scored incorrectly due to poor resolving power of the compound microscope, two samples were scored according to the same criteria but using optical sections (less than 1 μm thick) generated by confocal microscopy (Fig. 3). Calculations and graphs were generated with Microsoft Excel and R (www.r-project.org).

Table 1. Primary antibodies used in this study

Antigen	Species	Source	Catalog number	Dilution
Amylase	Sheep	BioGenesis	0480-0104	1:2500
Amylase	Rabbit	Sigma	A8273	1:1000
Cytokeratin-19	Rat	Developmental Studies Hybridoma Bank	TROMA-3	1:50
Cpa1	Goat	R&D systems	AF2765	1:2000
C-peptide (insulin)	Rabbit	Linco	4020-01	1:2500
C-peptide (insulin)	Goat	Linco	4023-01	1:5000
Cre	Mouse	Millipore	MAB3120	1:500
E-cadherin	Rat	Zymed/Invitrogen	13-1900	1:2000
GFP	Rabbit	Abcam	ab290	1:4000
GFP	Goat	Rockland	600-101-215	1:2500
Glucagon	Rabbit	Zymed / Invitrogen	18-0064	1:250
Glucagon	Guinea pig	Linco	4031-01F	1:2500
Hes1	Rabbit	Nadean Brown (University of Cincinnati, OH, USA)		1:1000
Ki67	Rabbit	Vector labs	VP-RM04	1:150
Pdx1	Guinea pig	Chris Wright (Vanderbilt University, TN, USA)		1:10,000
PECAM	Rat	BD Pharmingen	553370	1:125
Somatostatin	Goat	Santa Cruz	sc-7819	1:500

RESULTS

Hes1 expression and gene targeting

To extend previous studies of *Hes1* expression (Apelqvist et al., 1999; Esni et al., 2004; Jensen et al., 2000; Lammert et al., 2000), we performed Hes1 immunostaining on pancreata of different embryonic stages, spanning major developmental transitions. At E11.5, when most cells are undifferentiated progenitors, we found widespread but non-uniform expression of Hes1 in the epithelium (Fig. 1A), consistent with previous immunostaining and in situ hybridization studies (Jensen et al., 2000; Lammert et al., 2000; Murtaugh et al., 2005; Nakhai et al., 2008). We observed Hes1 downregulation from E13.5, the onset of the 'secondary transition' wave of acinar and β-cell differentiation (Gittes, 2009; Pictet and Rutter, 1972). Hes1 was initially mosaic throughout the epithelium (Fig. 1B), partly overlapping with the 'tip cell' marker carboxypeptidase A1 (Cpa1) (see Fig. S3A in the supplementary material) (Zhou et al., 2007), but became increasingly confined to Cpa1-negative ductal and centroacinar cells at later stages (Fig. 1C-D; Fig. S3B,C in the supplementary material). A similar restriction of Hes1 from tip cells has been seen previously (Esni et al., 2004), and the late ductal localization prefigures its expression in the adult (Miyamoto et al., 2003; Stanger et al., 2005). Antibody specificity was indicated by the lack of staining in *Hes1*-deficient embryos (generated as described below) (see Fig. S3D,E in the supplementary material). Together, these data unify previous studies, and support the hypothesis that Hes1 expression marks early pancreatic progenitors (Esni et al., 2004; Jensen et al., 2000).

In order to follow the fate of *Hes1*$^{+}$ cells, we engineered a *Hes1*^{CreERT2} allele (henceforth, *Hes1*^{C2}) by replacing most of the coding region with *CreERT2*, a tamoxifen-inducible recombinase (Feil et al., 1997) (Fig. 1E; Fig. S1 in the supplementary material). *Hes1*$^{C2/+}$ animals were viable and fertile, while most *Hes1*$^{C2/C2}$ embryos died between E12.5 and E13.5 (data not shown), as described for *Hes1* knockouts (Ishibashi et al., 1995).

To confirm that *Hes1*^{C2} was active in *Hes1*-expressing cells, we immunostained for Hes1, Cre and *lacZ* in E12.5 embryos double-transgenic for *Hes1*^{C2} and the lineage reporter *R26R*^{LacZ} (Soriano, 1999), which had received tamoxifen (TM) by maternal gavage at E9.5. We observed close overlap between Cre and Hes1 (see Fig. S4A-F in the supplementary material), indicating that *Hes1*^{C2} recapitulates endogenous *Hes1* expression. Furthermore, the *lacZ* lineage marker was widely expressed in the pancreatic epithelium, including by numerous cells that continue to express Hes1 (see Fig. S4G-I, arrowheads, in the supplementary material). In organ cultures, 4-hydroxytamoxifen treatment of dorsal pancreatic buds from E11.5 embryos double-transgenic for *Hes1*^{C2} and the lineage reporter *R26R*^{EYFP} (Srinivas et al., 2001) induced widespread epithelial EYFP expression. Labeling was almost abolished by pre-treatment with the γ-secretase inhibitor DAPT (Dovey et al., 2001) (see Fig. S5 in the supplementary material), indicating that *Hes1*^{C2} expression requires Notch activity. Together, these results indicate that *Hes1*^{C2} is expressed and regulated in the same way as endogenous *Hes1*, and that *Hes1*^{C2} can be used to follow the fate of Notch-responsive cells.

Progressive lineage restriction of embryonic *Hes1*$^{+}$ pancreatic progenitor cells

We performed additional crosses between *Hes1*^{C2} and *R26R*^{LacZ}, administered a single 2 mg tamoxifen dose to pregnant females between E9.5 and E15.5, and analyzed *lacZ* expression in the pancreas and gut at E17.5 (n=3-5 embryos per treatment group). No recombination was detected in the absence of tamoxifen (Fig. 2A,F). The labeling frequency in the pancreas was highest (~25%) when tamoxifen was given at E9.5 (Fig. 2B), and decreased with later treatment: ~12% *lacZ*$^{+}$ with TM at E11.5, ~8% at E13.5 and ~5% at E15.5 (Fig. 2C-E). [In this and other experiments, we analyzed multiple sections spaced throughout the specimen, to avoid errors due to stochastic variations in labeling efficiency (see Fig. S2 in the supplementary material).] The *Hes1*^{C2} labeling pattern agrees with the downregulation of endogenous Hes1 expression (Fig. 1A-D), and was reproduced using the *R26R*^{EYFP} reporter (data not shown). In contrast to the pancreas, liver labeling by *Hes1*^{C2} increased with later tamoxifen administration, particularly in cells adjacent to the portal veins (Fig. 2F-J). Almost all labeled cells in the liver expressed the ductal plate markers CK19 and E-cadherin (see Fig. S6 in the supplementary material and data not shown), consistent with studies showing that Notch and Hes1 promote intrahepatic bile duct development (Antoniou et al., 2009; Geisler et al., 2008; Kodama et al., 2004; Lozier et al., 2008; Zong et al., 2009).

To determine the fate of *Hes1*^{C2}-labeled cells in the pancreas, we stained these specimens for endocrine and exocrine differentiation markers, and calculated the fraction of labeled (*lacZ*$^{+}$) cells expressing each marker. Scoring over 1000 *lacZ*$^{+}$ cells in each experimental group (see Table S1 in the supplementary material), we found that *Hes1*$^{+}$ cells labeled at E9.5 generate both endocrine and exocrine progeny, with roughly one-third of all *lacZ*$^{+}$ cells co-expressing insulin (β-cells) or glucagon (α-cells), and the remainder comprising amylase^{+} acinar and DBA^{+} duct cells (Fig. 3A-D,I-J). [Note that although these counts were derived from images taken on a compound microscope, we obtained essentially identical numbers with images generated by confocal microscopy

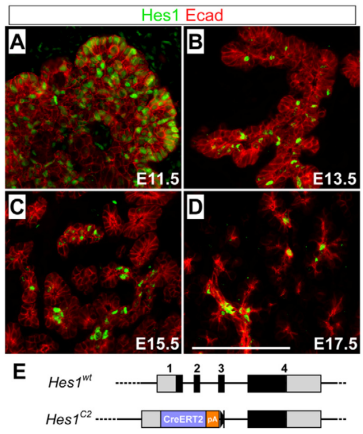

Fig. 1. Hes1 expression and targeting. (**A-D**) Staining for Hes1 (green) and the epithelial marker E-cadherin (red), in embryonic pancreata of the indicated stages. Scale bar: 100 μm. (**E**) Schematic of wild-type and targeted *Hes1*. The wild-type *Hes1* locus is depicted at the top (exons boxed and numbered, UTRs in grey and coding regions in black), for comparison with *Hes1*^{C2}, in which the *CreERT2* gene and bovine growth hormone polyadenylation signal (pA) are placed in-frame with the *Hes1* start codon, replacing much of the ORF.

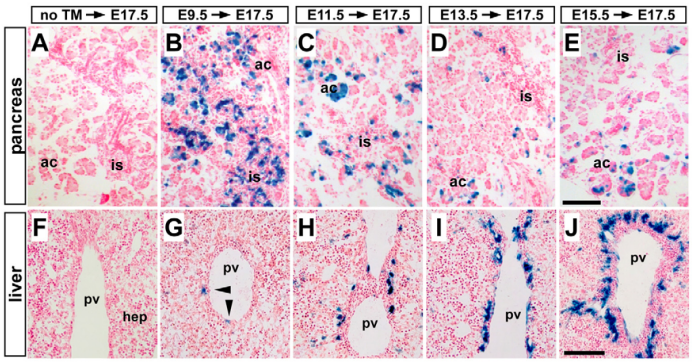

Fig. 2. Dynamic contribution of *Hes1*$^{+}$ cells to developing pancreas and liver. X-gal-stained sections of pancreas (**A-E**) or liver (**F-J**) from E17.5 *Hes1*$^{C2/+}$; *R26R*$^{LacZ/+}$ embryos that received no tamoxifen, or that received a single TM dose between E9.5 and E15.5. *lacZ*$^{+}$ cells stain blue, and sections are counterstained with Nuclear Fast Red. No recombination occurs without TM (A,F), whereas TM at E9.5 labels many pancreatic acinar (ac) and islet (is) cells (B). Pancreatic labeling declines with later TM treatment (C-E), whereas the opposite pattern is observed in the liver, where *lacZ*$^{+}$ cells are found near the portal veins (pv, arrowhead) (G-J). Few hepatocytes (hep) are labeled at any stage. Scale bars: 100 μm.

(Fig. 3I-J).] This finding implies that early *Hes1*$^{+}$ cells are multipotent, a conclusion supported by a low-dose clonal labeling approach, previously used to demonstrate tip cell multipotency (Zhou et al., 2007) (see Fig. S7 in the supplementary material). Together, these results are consistent with Notch signaling through *Hes1* to maintain early multipotent progenitors.

Later *Hes1*$^{+}$ cells continued to generate exocrine progeny, while appearing to lose endocrine differentiation capacity: almost no α-cells were labeled by TM treatment at E13.5, and β-cell labeling approached zero at E15.5 (Fig. 3E-J). Reduced islet contribution was also seen when comparing E13.5-labeled pancreata, 2 weeks after birth, with those labeled at E9.5 (Fig. 3K-N). Delivery of live pups in these experiments required lower tamoxifen doses, which resulted in decreased labeling overall but did not affect the distribution of labeled cells among differentiated lineages. The fact that labeling efficiency was uncoupled from label distribution implies that *Hes1*^{C2} drives recombination within a cell population of relatively homogeneous potential for endocrine, duct and acinar differentiation, with the proportion of labeled cells depending on TM dose. We cannot exclude the existence of cells expressing *Hes1* at levels too low for labeling by *Hes1*^{C2}, the developmental potential of which might differ from those observed here. Nonetheless, our results suggest that *Hes1* expression shifts from multipotent to exocrine-restricted progenitors (Fig. 3O), consistent with *Hes1* having to turn off before the pro-endocrine gene *Neurog3* can turn on (Jensen et al., 2000; Lee et al., 2001).

Ectopic Notch activation blocks differentiation of early but not late *Hes1*$^{+}$ cells

The wave of acinar and islet cell differentiation that occurs at the secondary transition coincides with *Hes1* downregulation (Fig. 1A-D), and we have previously shown that artificially preventing Notch downregulation blocks this differentiation process (Murtaugh et al., 2003). This was achieved by crossing the pan-pancreatic driver *Pdx1Cre* (Gu et al., 2002) to *Rosa26*$^{Notch1IC\text{-}IRES\text{-}GFP}$ (henceforth, *Rosa26*^{NIC}), which drives co-expression of activated mouse Notch1 and GFP (Murtaugh et al., 2003) (Fig. 4A,G). To determine whether early and late *Hes1*$^{+}$ cells are similarly susceptible to Notch, we crossed *Hes1*^{C2} to *Rosa26*^{NIC}.

Pregnant females received a single 2 mg TM dose between E9.5 and E15.5, and double-transgenic offspring were analyzed at E17.5 (n=2 or 3 per timepoint, see Table S1 in the supplementary material). After TM at E9.5 or E11.5, GFP^{+} cells (expressing activated Notch) formed cystic structures lacking endocrine or acinar marker expression (Fig. 4B-C,H-I). This phenotype resembled that obtained with *Pdx1Cre* (Fig. 4A,G), and agrees with early *Hes1*$^{+}$ cells representing multipotent, Notch-sensitive progenitors. The GFP^{+} epithelia stained with DBA lectin, which marks mature ducts as well as embryonic progenitors (Kobayashi et al., 2002), but their cystic morphology distinguished them from the narrow and highly-branched ducts normally present at these stages (Fig. 4G-K).

Although *Hes1*^{C2} labeled very few islet cells at E13.5 or E15.5 (Fig. 3I), *Rosa26*^{NIC} prevented even this low level of islet differentiation (Fig. 4D,E). By contrast, exocrine differentiation of E13.5-E15.5 *Hes1*$^{+}$ cells appeared to be partially Notch resistant, as GFP^{+} cells were found integrated into normal acini and ducts (Fig. 4J-K). To determine if the blunted effects of late *Rosa26*^{NIC} activation were secondary to the overall decrease in *Hes1*^{C2} labeling efficiency (Fig. 2), we repeated E9.5 treatment with a lower tamoxifen dose (0.5 mg), to activate fewer cells. As previously, the rare GFP^{+} cells observed in this experiment formed abnormal cystic tubules (Fig. 4F,L), suggesting that early Notch activation can disrupt exocrine differentiation without a 'critical mass' of affected cells.

When *Rosa26*^{NIC} is activated by *Pdx1Cre*, all cells exhibit a Pdx1^{high} 'trapped progenitor' phenotype (Murtaugh et al., 2003) (see Fig. S8A,D in the supplementary material). When Notch was activated by *Hes1*^{C2} at E15.5, GFP^{+} cells were negative for Pdx1, which instead was expressed only by β-cells (see Fig. S8C,F in the supplementary material). Cells in which *Rosa26*^{NIC} was induced at E9.5 exhibited modest Pdx1 upregulation (see Fig. S8B,E in the supplementary material), suggesting that they retained a partial progenitor-like identity (Fig. 4N).

Further analysis of late-induced *Hes1*$^{C2/+}$; *Rosa26*$^{NIC/+}$ pancreata revealed that Notch activation at E13.5 caused most GFP^{+} cells to adopt a ductal rather than acinar fate, whereas after E15.5 activation the proportions were reversed (Fig. 4M). The latter resembles the wild-type distribution observed with *R26R*^{LacZ} (Fig. 3J), suggesting that Notch activation at E15.5

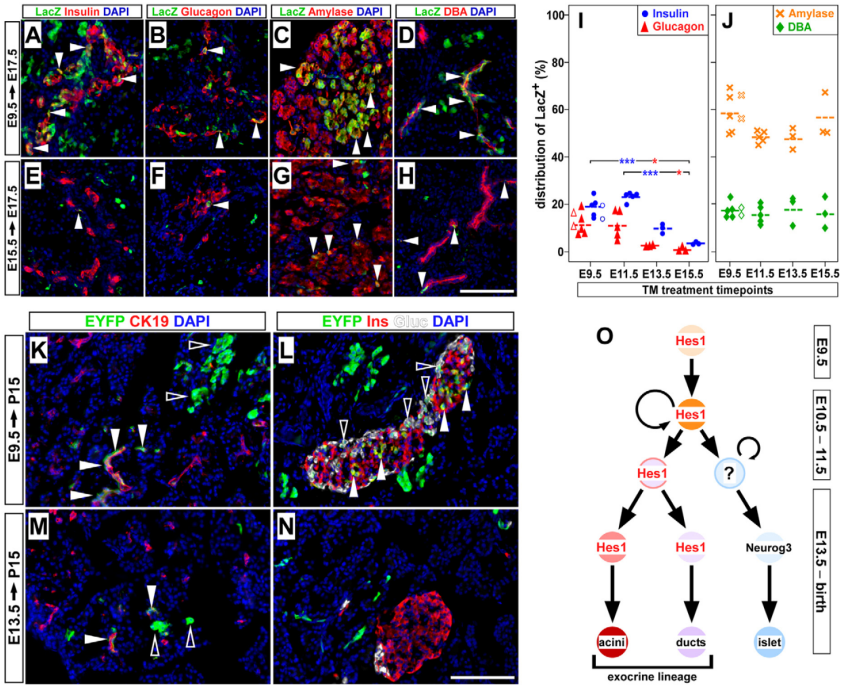

Fig. 3. Shift in differentiation potential of *Hes1*$^{+}$ cells during pancreas development. (**A-H**) *Hes1*$^{C2/+}$; *R26R*$^{LacZ/+}$ pancreata were TM labeled between E9.5 and E15.5, and analyzed at E17.5 (see Fig. 2). Staining for *lacZ* (green) and endocrine (insulin or glucagon) or exocrine (amylase or DBA) markers (red); nuclei stained with DAPI (blue). White arrowheads indicate *Hes1*^{C2}-labeled cells, which are most abundant following E9.5 labeling. Scale bar: 100 µm. (**I**,**J**) Quantitative distribution of *lacZ*$^{+}$ cells at E17.5, after TM treatment at indicated stages. Each point represents a single pancreas, in which at least five fields (300-1500 *lacZ*$^{+}$ cells) were scored for the fraction of *lacZ*$^{+}$ cells expressing the indicated marker: insulin (blue circle), glucagon (red triangle), amylase (orange X) or DBA (green diamond). Means are indicated by broken lines. Open points indicate data obtained using confocal microscopy. **P*<0.05 and ****P*<0.0005, by Tukey's HSD test. (**K-N**) *Hes1*$^{C2/+}$; *R26R*$^{EYFP/+}$ mice received TM in utero at E9.5 or E13.5 and were analyzed at P15 for co-expression of EYFP (green) with CK19 (left, red), insulin (right, red) or glucagon (right, white). *Hes1*$^{+}$ cells labeled at either stage contribute to ducts and acini (K,M, closed and open arrowheads), whereas only early *Hes1*$^{+}$ cells make significant contribution to adult β- and α-cells (L, closed and open arrowheads). Scale bar: 100 µm. (**O**) Early *Hes1*$^{+}$ cells appear to be multipotent, but become restricted to the exocrine lineage after E13.5, concomitant with the secondary transition.

does not perturb exocrine differentiation, while E13.5 activation drives bipotent progenitors toward a duct fate (Fig. 4N). Activated Notch can also respecify endocrine precursors to ducts (Greenwood et al., 2007), and this pro-ductal activity may underlie the pathological effects of Notch in pancreatic cancer (De La O et al., 2008).

Lineage tracing *Hes1*$^{+}$ cells in the adult pancreas and intestine

The question of whether pancreatic progenitor cells persist after birth is a matter of long-standing controversy, particularly with respect to adult differentiation of β-cells. Although several lineage-tracing studies indicate that adult duct and acinar cells do not generate new β-cells in the resting or regenerating pancreas (Desai et al., 2007; Kopinke and Murtaugh, 2010; Solar et al., 2009), these have not excluded the existence of specialized progenitor cells. Centroacinar cells (CACs), in particular, have been proposed to behave as β-cell progenitors (Hayashi et al., 2003; Nagasao et al., 2003; Rovira et al., 2010). CACs express higher levels of *Hes1* than do other exocrine cells (Miyamoto et al., 2003; Stanger et al., 2005), and we used *Hes1*^{C2} to determine whether these or other *Hes1*$^{+}$ cells behave as adult stem or progenitor-like cells.

To identify *Hes1*-expressing cells in the adult pancreas, we administered 10 mg tamoxifen to 2-month-old (P60) *Hes1*$^{C2/+}$; *R26R*$^{EYFP/+}$ mice (n=4), and analyzed EYFP expression ~48 hours later. Consistent with prior studies of Hes1 expression, we found EYFP labeling of approx. one-quarter of CK19^{+} centroacinar cells (Fig. 5A), as well as a lesser proportion of labeled cells within larger ducts (Fig. 5B). We also observed a small fraction of EYFP^{+} acinar cells, suggesting *Hes1* expression by rare, differentiated acinar cells (Fig. 5C). To follow the longer-term fate of adult (P60) *Hes1*$^{+}$ cells, we compared quantitatively the labeling obtained at 7 days post-TM,

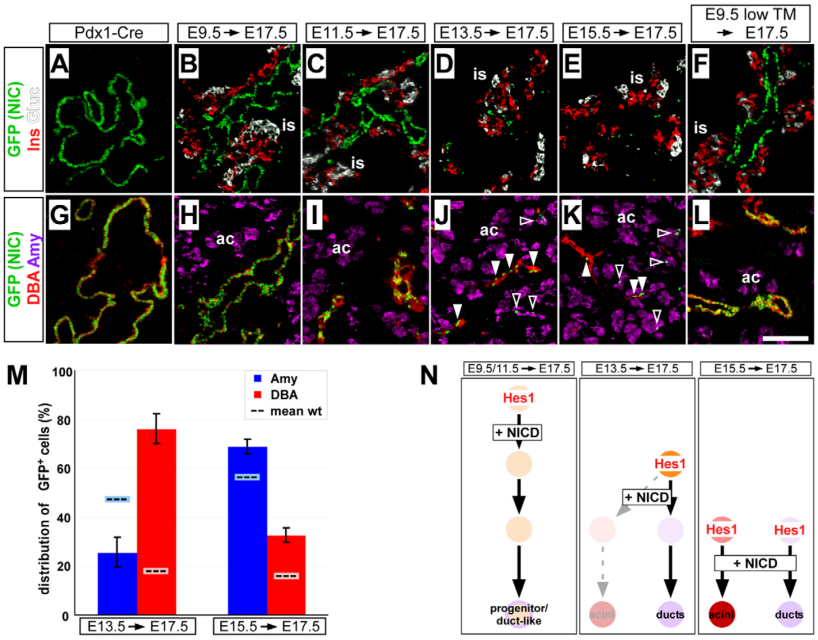

Fig. 4. Ectopic Notch activation blocks differentiation of early but not late *Hes1*$^+$ cells. (**A-L**) *Rosa26*^{NIC} was activated by *Pdx1Cre* or by *Hes1*$^{C2/+}$ following TM treatment between E9.5 and E15.5. E17.5 pancreata were stained for co-expression of GFP (green), marking *Rosa26*^{NIC} expression, with the endocrine markers insulin and glucagon (top, red and white) or exocrine markers DBA and amylase (bottom, red and purple). *Rosa26*^{NIC} activation in *Pdx1*$^+$ or E9.5 *Hes1*$^+$ cells blocks islet and acinar differentiation, and induces DBA$^+$ cysts (A,B,G,H). Lower-dose activation of *Hes1*^{C2} at E9.5 induces similar GFP$^+$ cysts (F,L). With later stage activation, GFP$^+$ cells assume an increasingly normal appearance (C-E,I-K), including integration into normal ducts and co-expression of amylase (closed and open arrowheads). Scale bar: 100 μm. (**M**) Quantitative distribution of GFP$^+$ cells among amylase$^+$ (blue) and DBA$^+$ (red) cells at E17.5, after *Rosa26*^{NIC} activation at E13.5 or E15.5 (~100 GFP$^+$ cells scored per sample). Broken lines indicate the normal distribution of *Hes1*^{C2}-labeled cells after TM treatment at these stages (from Fig. 3J). Notch activation at E13.5 promotes duct development, whereas activation at E15.5 does not perturb normal exocrine differentiation of *Hes1*$^+$ cells. Results are mean±s.e.m. (**N**) Notch activation in early (E9.5-E11.5) *Hes1*$^+$ progenitor cells (light orange) prevents normal differentiation and induces a progenitor/duct-like phenotype. At E13.5, Notch promotes mature duct development, whereas activation at E15.5 does not affect differentiation.

reflecting the initial differentiation state of *Hes1*$^+$ cells, to that observed after a 2-month 'chase', in which time cells might have adopted new fates. As in the 48-hour chase experiment, we detected acinar, duct and CAC labeling after 7 days, which persists at 2 months (Fig. 5D-G). Scoring the labeling index of each differentiated cell type (n=3-10 mice analyzed per condition) (see Table S2 in the supplementary material), we found that ~1% of acinar cells were EYFP$^+$ at each timepoint [1.4±0.3% (s.e.m.) at 7 days, 1.3±0.3% at 2 months; Fig. 5H]. By contrast, the labeling index of duct cells (defined here as CK19$^+$ epithelial cells not embedded within acini) increased by roughly twofold, from 8.0±0.6% EYFP$^+$ at 7 days to 15.5±0.6% EYFP$^+$ after 2 months (Fig. 5H). This might indicate that *Hes1* marks a subpopulation of proliferating duct cells, consistent with a mitogenic role for Notch in this lineage (Golson et al., 2009). Regarding centroacinar cells specifically, we found a similar labeling index at both timepoints (27.0±2.9% EYFP$^+$ at 7 days, 26.5±0.9% EYFP$^+$ at 2 months). The relative labeling indices of ducts and CACs raises an alternative explanation of why duct labeling increases over time, namely that expansion of the ductal tree is driven by descent from CACs. Although anatomically plausible, this hypothesis requires work beyond the scope of this study.

Unexpectedly, we also detected *Hes1*^{C2}-labeled cells within islets. In islets and throughout the pancreas and other organs, *Hes1*^{C2} labeled numerous endothelial cells (~20% in all experiments) (see Fig. S9 in the supplementary material and data not shown), which we have not analyzed further. With respect to endocrine cells, we did not observe a single *Hes1*^{C2}-labeled β-cell in these experiments, out of over 2000 insulin$^+$ cells scored at each timepoint (see Table S2 and Fig. S9A-B in the supplementary material). We did find rare glucagon$^+$ α-cells marked by *Hes1*^{C2} at both timepoints (3.2±0.4% EYFP$^+$ at 7 days post-TM, 5.7±1.1% at 2 months) (see Fig. S9C-D in the supplementary material), as well as after very short chase periods (12-24 hours post-TM, data not shown). Although the increased α-cell labeling with time is statistically significant (P<0.05), its biological relevance is unclear: it could indicate rare neogenesis

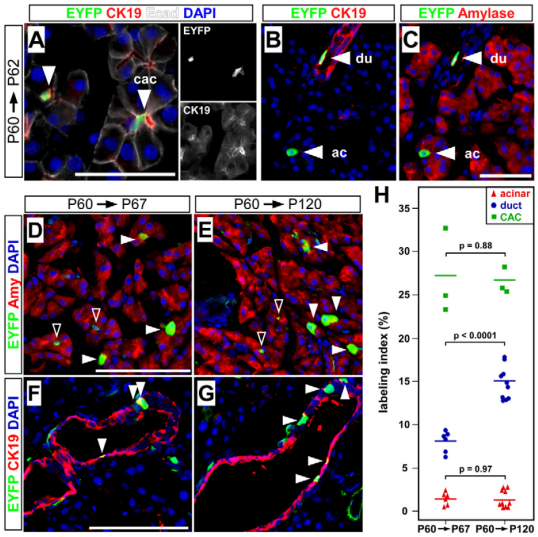

Fig. 5. *Hes1* expression and lineage tracing in the adult exocrine pancreas. Adult (P60) *Hes1*$^{C2/+}$; *R26R*$^{EYFP/+}$ mice were treated with tamoxifen and analyzed for EYFP expression (green) after 2-60 days. (**A**) After short-term labeling, EYFP is expressed by numerous CK19^{+} (red)/E-cadherin^{+} (white) centroacinar cells (cac). Right, single-channel EYFP and CK19 staining. (**B**,**C**) After short-term labeling, EYFP is also detected in CK19^{+} duct (du) cells (B, red) and amylase^{+} acinar (ac) cells (C, red). (**D**,**E**) A similar fraction of labeled acinar (white arrowheads) and centroacinar cells (open arrowheads) is seen after 7- or 60-day chase periods. (**F**,**G**) Between 7 and 60 days post-TM, the fraction of EYFP-labeled duct cells (arrowheads) appears to expand. Scale bars: 50 μm in A-C; 100 μm in D-G. (**H**) Quantifying labeled cells as a fraction of all acinar (red triangles), duct (blue circles) or centroacinar cells (green squares). Each point represents the labeling index of at least five fields from a single pancreas; mean labeling indices (across multiple pancreata) are indicated by horizontal lines. Acinar and CAC labeling does not change over time, whereas that of duct cells increases. *P*-values are determined by Tukey's HSD test.

from the more highly labeled ducts or CACs, although this has not been seen in previous experiments (Kopinke and Murtaugh, 2010; Solar et al., 2009). Alternatively, and consistent with α-cells being labeled at short chase periods, *Hes1* could be expressed by rare α-cells and mark a more proliferative subset of this population. Adult α-cells dynamics have received less attention than those of β-cells, although tools now exist to determine whether significant numbers of adult α-cells are born outside the islet (Thorel et al., 2010).

The lack of β-cell labeling suggests that *Hes1*$^{+}$ cells do not behave as adult precursors for this cell type. Our TM treatment regimen appeared to capture most *Hes1*$^{+}$ cells in the adult pancreas: with respect to centroacinar cells, a higher dose of tamoxifen (3×10 mg, over 3 days) conferred no more labeling than our standard 1×10 mg dose (24.9±0.1% EYFP^{+} at 1 month post-treatment, *n*=2). We propose that *Hes1* expression marks only a subset of CACs in the adult pancreas, which does not contribute to β-cells in vivo.

Independent evidence for the efficiency of adult *Hes1*^{C2} labeling comes from the adult intestine, in which we found that *Hes1*^{C2} does mark stem cells. Low dose (2 mg) tamoxifen treatment of adult (P60) *Hes1*$^{C2/+}$; *R26R*$^{EYFP/+}$ mice (*n*=2) revealed labeling, within 12 hours, of single cells in the crypt base region as well as just above the crypts (Fig. 6A,B). The location of the former cells, representing approx. two-thirds of all EYFP^{+} cells in the intestinal epithelium, agreed with previous *Hes1* expression studies (Jensen et al., 2000; Schroder and Gossler, 2002), whereas the latter may represent transit-amplifying cells in which Notch inhibits secretory lineage specification (Crosnier et al., 2006). After a long-term chase (30 days, *n*=2), we found entire crypt-villus units expressing EYFP, suggesting labeling of intestinal stem cells (Fig. 6C,D). We also found that *Hes1*^{C2}-expressing cells, like bona fide intestinal stem cells (Barker et al., 2009; Sangiorgi and Capecchi, 2008; Zhu et al., 2008), were susceptible to transformation by activated β-catenin [using a *Ctnnb1*$^{lox(ex3)}$ gain-of-function allele (Harada et al., 1999)] (Fig. 6E,F). Importantly, a single 5 mg TM dose was sufficient to label most crypt-villus units along the intestinal tract of *Hes1*$^{C2/+}$; *R26R*$^{LacZ/+}$ mice (*n*=2) after a 6-month chase. Labeling was highest in the duodenum (~90% of crypts), and decreased posteriorly to ~50% labeling in the colon (Fig. 6G-J), frequencies that compare favorably with those of other intestinal stem cell Cre drivers (Barker et al., 2007; Sangiorgi and Capecchi, 2008; Zhu et al., 2008). This high efficiency implies that a 5 mg TM dose was sufficient to recombine most *Hes1*$^{+}$ cells in the intestine; assuming a similar dose-response relationship in the pancreas, our 10 mg TM treatment regimen should also have labeled most *Hes1*$^{+}$ cells in this organ. Taken together, our results highlight the contrasting cellular dynamics of the adult pancreas and intestine, and suggest that *Hes1*^{C2} might be useful to mark and manipulate adult stem cells when they exist in other organs.

Hes1^{CreERT2}-labeled cells do not contribute to β-cells after injury

Although β-cell neogenesis may not be required in the healthy adult pancreas, it might be induced by injury, as in the case of pancreatic duct ligation (PDL) (Inada et al., 2008; Xu et al., 2008). PDL causes inflammation and acinar cell apoptosis distal to the ligation site (Scoggins et al., 2000; Watanabe et al., 1995), which is accompanied by a local increase in β-cell numbers and apparent reappearance of *Neurog3*$^{+}$ β-cell precursors (Wang et al., 1995; Xu et al., 2008). We therefore sought to determine whether *Hes1* marks cells capable of β-cell neogenesis in this model.

Ligation of the dorsal (splenic) pancreas lobe was performed as described by others (Scoggins et al., 2000; Solar et al., 2009; Watanabe et al., 1995; Xu et al., 2008); a full description of our observations in this model will be submitted elsewhere. At 7 days after surgery, acinar cells in the ligated region were completely replaced by fibro-inflammatory cells and epithelial tubules

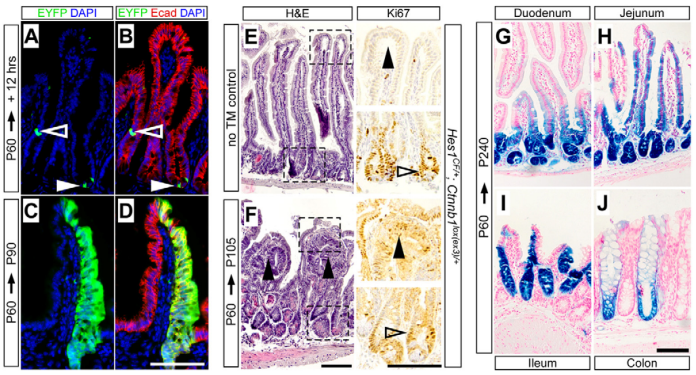

Fig. 6. *Hes1* expression marks intestinal crypt stem cells. (**A-D**) Adult (P60) *Hes1*$^{C2/+}$; *R26R*$^{EYFP/+}$ mice received a single tamoxifen dose, and were stained for labeling of the ileum epithelium (EYFP, green; E-cadherin, red) after 12 hours or 30 days. Short-term labeling (A,B) marks cells in the basal crypt (closed arrowhead) and at the crypt-villus junction (open arrowheads). After a 30-day chase (C,D), labeling encompasses the entire crypt-villus unit, indicating stem cell labeling. (**E**,**F**) P60 *Hes1*$^{C2/+}$; *Ctnnb1*$^{lox(ex3)/+}$ mice were left untreated (E) or administered a single tamoxifen dose (F), and analyzed after 45 days. Hematoxylin and Eosin staining reveals normal morphology of untreated intestine, whereas the small intestines of TM-treated mice exhibit numerous microadenomas (arrowheads) accompanying general tissue disorganization. Outlines indicate areas stained for Ki67 on adjacent sections, which reveal TM-induced expansion of the proliferative compartment from crypts (open arrowheads) to more distal epithelium (closed arrowheads). (**G-J**) *Hes1*$^{C2/+}$; *R26R*$^{LacZ/+}$ mice received a single 5 mg TM dose at P60 and were chased for 180 days before analysis by whole-mount X-gal staining of specific intestinal segments. Uniformly *lacZ*$^{+}$ crypts are detected in all segments, at a decreasing frequency from anterior to posterior. Staining of distal villi was inconsistent (e.g. G) owing to poor penetration of substrate. Scale bars: 100 μm.

(Fig. 7A,B). These epithelia presented suggestive evidence of neogenesis, including association with small β-cell clusters, upregulation of Pdx1, and re-expression of *Neurog3* (Fig. 7E-G and data not shown).

To follow *Hes1*$^{+}$ cells, we administered TM to *Hes1*$^{C2/+}$; *R26R*$^{EYFP/+}$ mice either 1 month pre-surgery (to mark *Hes1*$^{+}$ cells in the healthy pancreas; *n*=8) or 3 days post-surgery (to capture progenitor-like cells upregulating *Hes1* after injury; *n*=4), and analyzed EYFP expression 7 days after injury. The 7-day timepoint was chosen for consistency with previous studies indicating a doubling of β-cell mass at this stage, possibly owing to ductal neogenesis (Solar et al., 2009; Wang et al., 1995; Xu et al., 2008). From each mouse, we analyzed 8-10 sections spaced evenly throughout the ligated region (see Fig. S2 in the supplementary material), manually scanning the entire area of each section for EYFP^{+}/insulin^{+} cells (encompassing ~10,000 insulin^{+} cells scanned per ligated pancreas). We did not find a single *Hes1*^{C2}-labeled β-cell in any pancreas, regardless of labeling strategy (Fig. 7C,D and data not shown). We did observe widespread labeling of E-cadherin^{+} and CK19^{+} epithelial complexes in ligated pancreata [~15-20%, a labeling index similar to that of duct and centroacinar cells in the absence of injury (Fig. 5H)], in both TM treatment groups (Fig. 7C,D) (see Table S2 in the supplementary material and data not shown). Although insulin^{+} cells were frequently associated with these complexes, they were always unlabeled (Fig. 7E). These results suggest that *Hes1*^{C2} does not mark cryptic or injury-induced β-cell progenitors in the adult. Although these findings do not exclude the possibility of β-cell differentiation from cultured centroacinar cells in vitro (Rovira et al., 2010), or from *Hes1*-negative cells in vivo, they agree with an independent finding that ducts do not generate β-cells after PDL (Solar et al., 2009).

DISCUSSION

Hes1 is a major Notch target in diverse tissues (Kageyama et al., 2007), and we use its expression as a 'tag' to determine where and when Notch is active in the pancreas. In the embryonic pancreas, our results suggest that early *Hes1*$^{+}$ cells are multipotent progenitors, the differentiation of which is inhibited by Notch, whereas late *Hes1*$^{+}$ cells are exocrine restricted, and respond to elevated Notch levels by becoming ducts (Fig. 3H; Fig. 4F). Our findings in the adult pancreas suggest that *Hes1* expression is restricted to differentiated cells, most abundant within the centroacinar population, and that *Hes1*$^{+}$ cells do not normally behave as cryptic or facultative stem cells for endocrine β-cells. *Hes1*^{C2} provides a novel tool with which to analyze embryonic and adult cells in the pancreas, and our studies of the adult intestine suggest that it might be widely useful in marking and manipulating adult stem cells.

Hes1 lineage and Notch function in the embryonic pancreas

Our results confirm and significantly extend prior studies of Notch-responsive cells in the embryonic pancreas. For example, mapping Notch1 receptor activation in vivo, via *N$_1$IP-Cre*, reveals scattered labeling throughout the exocrine and endocrine pancreas (Vooijs et al., 2007). Although *N$_1$IP-Cre* identifies the range of cell types that had experienced Notch1 signaling at some prior stage, it cannot determine when that signaling occurred. As our CreERT2 approach allowed us to mark cells expressing *Hes1* at specific developmental stages, we could show that most islet-fated cells had received Notch signals only before the 'secondary transition', a wave of endocrine differentiation spanning ~E13.5 to birth in the mouse (Herrera et al., 1991; Pictet and Rutter, 1972).

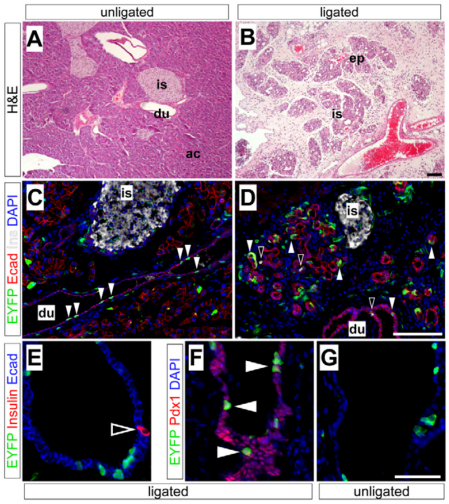

Fig. 7. No detectable β-cell neogenesis from *Hes1*$^{+}$ cells after duct ligation. (**A**,**B**) Hematoxylin and Eosin-stained sections of wild-type pancreata, 7 days after duct ligation, reveals loss of acinar cells (ac) and expansion of epithelial nests (ep) specifically in the ligated region. Islets (is) are preserved. (**C-G**) *Hes1*$^{C2/+}$; *R26R*$^{EYFP/+}$ mice received TM 1 month pre-ligation, and were analyzed 7 days post-ligation by staining for EYFP (green), E-cadherin (C,D, red; E, blue), insulin (C,D, white; E, red) and Pdx1 (F,G, red). In the unligated region (C), the *Hes1* lineage encompasses duct (closed arrowheads), acinar and endothelial cells. In the ligated area (D), labeling is found in epithelial (closed arrowheads) and endothelial cells. Insulin^{+} cells in duct-like structures are unlabeled (D,E, open arrowheads). Pdx1 is upregulated in ligated ducts, including *Hes1*^{C2}-labeled cells (F), but remained undetectable in unligated ducts (G). Scale bars: 100 μm in A-D; 50 μm in E-G.

The fact that Hes1 can directly repress *Neurog3* (Lee et al., 2001), together with the excessive α-cell differentiation observed in *Hes1* mutants (Jensen et al., 2000), might suggest that *Hes1* downregulation is rate-limiting for endocrine specification. However, *Neurog3*$^{+}$ endocrine precursors continue to be generated throughout the secondary transition, with a peak at E15.5 (Gradwohl et al., 2000; Gu et al., 2002), and the majority of β-cells differentiate between E15.5 and birth (Herrera et al., 1991). Our results suggest that these cells must derive from progenitors that turn on *Neurog3* several days after having turned off *Hes1*, arguing that endocrine specification is not immediately induced upon Notch-*Hes1* downregulation. Indeed, the secondary transition appears to proceed normally in *Notch1*/*Notch2* double mutants (Nakhai et al., 2008), suggesting that Notch-independent mechanisms control the timing of *Neurog3* expression and islet differentiation in late embryogenesis.

Prior to the secondary transition, both *Cpa1*$^{+}$ tip cells and *Hnf1b*$^{+}$ ducts contain multipotent progenitors (Solar et al., 2009; Zhou et al., 2007). *Hes1* is expressed in and labels both tip cells and ducts during early pancreas development (see Fig. S3A in the supplementary material and data not shown), suggesting that it marks multipotent progenitors regardless of anatomical location. After E13.5, *Cpa1*$^{+}$ cells behave as acinar-restricted precursors, whereas *Hnf1b*$^{+}$ cells become restricted to islet and duct fates (Solar et al., 2009; Zhou et al., 2007). To reconcile these observations with our hypothesis that *Hes1* marks bipotent acinar/duct progenitors after E13.5, we suggest that acinar-restricted *Cpa1*$^{+}$ cells derive from bipotent *Hes1*$^{+}$ cells from ~E13.5-E15.5. Indeed, analysis of GFP perdurance in *Sox9-EGFP* transgenic embryos suggests that ducts give rise to acini through at least E14 (Seymour et al., 2008). That late duct-to-acinar differentiation was not observed with *Hnf1b-CreERT2* may reflect inefficient labeling by this transgene in utero (Solar et al., 2009), combined with the overall rarity of *Hes1*$^{+}$ progenitors at these stages (Figs 1, 2). In zebrafish, Notch is required for duct specification of exocrine-restricted progenitors (Yee et al., 2005), and our results suggest that Notch also promotes duct development in late mouse embryogenesis.

Hes1 expression and phenotypic plasticity in the adult pancreas

Notch is implicated in self-renewal of adult stem cells (Chiba, 2006), and *Hes1*^{C2} robustly labels stem cells in the intestinal crypts. Under conditions sufficient to label the majority of intestinal stem cells, however, we do not find evidence that *Hes1*^{C2} labels stem-like cells in the adult pancreas. Instead, we find that *Hes1*^{C2} is active in several mature cell types, of which centroacinar cells are the most highly labeled. Previous studies indicate that *Hes1* expression and Notch activity are highest in CACs, and lower in more proximal ductal elements (Miyamoto et al., 2003; Parsons et al., 2009; Stanger et al., 2005), closely paralleling the *Hes1*^{C2} labeling pattern. Although we have not obtained reliable Hes1 immunostaining in adult pancreata (data not shown), *Hes1*^{C2} labeling suggests that it is also expressed by rare differentiated α-cells and acinar cells. Whether Notch has a functional role in these cells remains to be determined. Importantly, we never observe *Hes1*^{C2} labeling of insulin^{+} β-cells, suggesting little or no contribution to these cells from the adult *Hes1* lineage.

This result appears inconsistent with the finding that CACs, isolated based on high aldehyde dehydrogenase activity, can give rise to β-cells and other cell types in vitro (Rovira et al., 2010). Interestingly, however, the cells isolated in that study expressed only low levels of *Hes1*, suggesting that they represent a distinct subpopulation of CACs. Indeed, we find that only approx. one-quarter of CACs are labeled by *Hes1*^{C2} using our standard tamoxifen dose, and that this proportion is not increased by a threefold higher dose. Our results therefore constitute in vivo evidence for heterogeneity within the duct and CAC compartments, and suggest that the *Hes1*$^{+}$ subpopulation does not normally give rise to β-cells.

Alternatively, the failure of *Hes1*^{C2} to label β-cells might reflect limitations imposed by the micro-environment of the mature pancreas, e.g. active Notch signaling reinforcing ductal fate, which could be removed in tissue culture or during regeneration. To address this, we adopted an injury model, pancreatic duct ligation, which has provided suggestive evidence of β-cell neogenesis from ductal progenitors (Wang et al., 1995; Xu et al., 2008). In rats and mice, PDL is reported to lead to a local doubling of β-cell mass within 1 week (Wang et al., 1995; Xu et al., 2008), together with inflammation, acinar cell apoptosis and ductal hyperplasia (Scoggins et al., 2000; Watanabe et al., 1995). At 7 days post-PDL, we find that *Hes1*^{C2}-labeled cells contribute to the abnormal ductal epithelium, but

not to β-cells located either in islets or within or near ducts. We note that identical results were obtained using *Hnf1b-CreERT2*, which labels cells throughout the ductal network (Solar et al., 2009), suggesting that new β-cells arise after PDL either from pre-existing β-cells, or from a duct subpopulation that expresses neither *Hnf1b* nor *Hes1* (Inada et al., 2008).

Our results do not exclude the possibility that CACs or other $Hes1^+$ cells could give rise to β-cells more than 7 days post-PDL, although further increases in β-cell mass beyond this timepoint have not been reported (Wang et al., 1995; Xu et al., 2008), and adipocyte infiltration at later stages may produce secondary effects on islets (Watanabe et al., 1995). It is also possible that other injury models might evoke β-cell differentiation from $Hes1^+$ cells, much as glucagon$^+$ α-cells can transform into β-cells after extreme β-cell loss, despite an otherwise absolute barrier to interconversion (Thorel et al., 2010). We also note that, as $Hes1^{C2}$ labels a minority of CACs, our study does not definitively test CAC differentiation potential. Several other CreERT drivers, particularly those with stringent tamoxifen dependence, have been shown to recombine only a minority of their putative target cells (Desai et al., 2007; Dor et al., 2004; Solar et al., 2009), raising the possibility of unlabeled subpopulations. Our results, however, suggest that $Hes1^{C2}$ does label most $Hes1^+$ cells in the adult, but that $Hes1^+$ duct and centroacinar cells cannot generate β-cells under the conditions studied here. In sum, $Hes1^{C2}$ provides a new tool to test the role of Notch-responsive cells in physiological and pathological conditions, and our studies raise the issue of whether Notch activity functionally subdivides pancreatic duct cells into those with and without progenitor-like potential.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

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CHAPTER 6

ONGOING NOTCH SIGNALING MAINTAINS PHENOTYPIC FIDELITY IN THE ADULT EXOCRINE PANCREAS

Introduction

Tissue maintenance during homeostasis or repair from injury is achieved through three potential mechanisms. In the classic stem cell model, both cell turnover and pathological cell loss are counterbalanced by continuous influx from dedicated adult stem cells. Another model involves replacement of lost cells by replication of pre-existing, fully differentiated cells. According to a hybrid "facultative stem cell" model, however, differentiated cells of one lineage can give rise to another, under specific circumstances such as injury (Yanger and Stanger, 2011). This model remains controversial in most tissues, for want of support by lineage tracing and/or evidence for molecular mechanism.

The mammalian pancreas experiences very little cell turnover during normal homeostasis, and most evidence to date indicates that its cell types are maintained by faithful replication of pre-existing cells. In the adult endocrine pancreas, replication is the main mode of generating new insulin-producing β-cells (reviewed in Pan and Wright, 2011). Replication also appears to be the mechanism by which acinar cells, belonging to the exocrine pancreas, are maintained during adult homeostasis and regeneration (Desai et al., 2007; Strobel et al., 2007). The cell most often proposed as an adult progenitor-

like cell in the pancreas is the centroacinar cell (CAC). CACs represent the terminal element of the ductal tree and are characterized by their central position within individual acinar rosettes (Ekholm et al., 1962). CACs are considered potential cells-of-origin for pancreatic cancer (Maitra and Hruban, 2008; Stanger et al., 2005), have been suggested to produce new β-cells following injury (Hayashi et al., 2003; Nagasao et al., 2003), and they exhibit progenitor-like behavior following isolation and culture *in vitro* (Rovira et al., 2010). Whether CACs actually behave as adult progenitor cells *in vivo* has remained controversial, as tools for lineage tracing these cells have been lacking until very recently.

Another feature of CACs, and to a lesser extent ducts, is that they express *Hes1*, a downstream target of the Notch signaling pathway (Kopinke et al., 2011; Miyamoto et al., 2003; Parsons et al., 2009; Stanger et al., 2005). Activation of this pathway involves juxtacrine interactions between ligands of the Delta/Serrate family and receptors of the Notch family, which trigger the protease-induced release and nuclear translocation of the Notch intracellular domain (NIC). Nuclear NIC binds the transcription factor Su(H)/CSL/RbpJκ (henceforth referred to as RbpJ), and coactivates target genes including the Hes/Hey family of transcriptional repressors (Kageyama et al., 2007; Kopan and Ilagan, 2009). Across multiple phyla and tissues, Notch signaling frequently acts to maintain "stemness" or to control binary cell fate decisions (Chiba, 2006). In the embryonic pancreas, hyperactivation of Notch inhibits exocrine acinar cell development and instead promotes progenitor maintenance (Esni et al., 2004; Hald et al., 2003; Murtaugh et al., 2003).

We have recently generated a new tamoxifen-inducible Cre line under the control of the *Hes1* promoter (*Hes1*^{CreERT2}, abbreviated *Hes1*^{C2}), which faithfully marks *Hes1*$^{+}$ CACs. By performing lineage tracing, we showed that *Hes1*-expressing CACs do not contribute to new β- or acinar cells during adult homeostasis. During embryogenesis, however, *Hes1*$^{+}$ cells represent bipotent exocrine progenitor in which ectopic Notch promotes duct specification at the expense of acinar fate (Kopinke et al., 2011). Thus, sustained Notch signaling in *Hes1*$^{+}$ CACs might restrain the full differentiation potential of these cells. In the current study, therefore, we aimed to challenge the system by disrupting Notch signaling specifically in *Hes1*-expressing cells, during normal homeostasis and after pancreatic ductal ligation (PDL). To examine further a potential role of CACs in acinar cell regeneration, we also performed *Hes1* lineage tracing after caerulein-induced pancreatitis. We find that acute loss of *RbpJ* in adult *Hes1*$^{+}$ CACs causes their rapid transdifferentiation into acinar but not islet cells, suggesting that loss of Notch activity uncovers a facultative acinar progenitor cell phenotype in CACs. In fact, we find also that wild-type Hes1-expressing CACs contribute to regeneration of acinar cells following acute pancreatitis. Our results provide the first evidence of an endogenous genetic program to control interconversion of cell fates in the adult pancreas.

Materials and methods

Mice

Hes1^{C2} (Kopinke et al., 2011), *R26R*^{EYFP} (Srinivas et al., 2001) and *RbpJ*^{lox} (Han et al., 2002) mice have been described previously. *Ptf1a*^{CreERT} mice were generated by

recombinase-mediated cassette exchange (Burlison et al., 2008), inserting the CreERT coding region into the first exon of *Ptf1a* (full details of this allele will be published elsewhere). *RbpJ*^{lox} mice, kindly provided by Tasuku Honjo (Kyoto University) and Sean Morrison (University of Michigan), were crossed to *Hprt*-Cre deletor mice (Tang et al., 2002) to generate a null (*RbpJ*$^{\Delta}$) allele. PCR genotyping for the floxed allele of *RbpJ* was performed as described (Han et al., 2002); for the null allele the following oligos were used: wt forward: 5'- TAACTATCTTGGAAGGC TAAAAT-3'; and mutant reverse: 5'- GCTTGAGGCTTGATGTTCTGTATTGC-3' (598 bp product).

Animal experiments

Tamoxifen (Sigma T-5648) was dissolved in corn oil, and administered by oral gavage at doses of 5 mg (*Ptf1a*^{CreERT}) or 10 mg (*Hes1*^{C2}) per mouse between 6-8 weeks of age. BrdU (Sigma) was dissolved in drinking water (1 mg/ml) and provided to mice ad libitum 3 days prior to and until 7 days after tamoxifen treatment. Pancreatic duct ligations were performed as described (Kopinke et al., 2011). Acute pancreatitis was induced in *Hes1*$^{C2/+}$*; R26R*$^{EYFP/+}$ mice by eight hourly i.p. injections of caerulein (Bachem; 0.1 μg/g in saline) per day over 2 days (Jensen et al., 2005). Control mice received the equivalent amount of saline alone. Serum amylase levels were monitored by obtaining blood samples one day before the first injection and 1 hour after the last. Amylase levels were measured on a VMax Kinetic microplate reader (Molecular Devices) using Infinity serum amylase reagent (Thermo-Fisher). All animal procedures were approved by the Institutional Animal Care and Use Committee of the University of Utah.

Staining and analysis

Immunostaining and analysis were performed as previously described (Kopinke et al., 2011; Kopinke and Murtaugh, 2010). The following primary antibodies were used: sheep anti-amylase 1:2500 (BioGenesis), rat anti-BrdU 1:2000 (Abcam), rabbit anti-cytokeratin-19 1:1500 (Ben Stanger), rat anti-cytokeratin-19 1:50 (Developmental Studies Hybridoma Bank), rabbit anti-cleaved Caspase3 1:1000 (Cell Signaling), rat anti-E-cadherin 1:2000 (Zymed), rabbit anti-GFP 1:4000 (Abcam), goat anti-GFP 1:2500 (Rockland), guinea pig anti-glucagon 1:2500 (Linco), rabbit anti-glucagon 1:2500 (Zymed), guinea pig anti-Insulin 1:2000 (Dako), rabbit anti-Ki67 1:150 (Vector labs) and rabbit anti-Ptf1a 1:800 (gift from Helena Edlund). All secondary antibodies (raised in donkey) were obtained from Jackson Immunoresearch. To detect Ki67 and BrdU by immunofluorescence, a 15 min DNAse I digestion (700 U/µl, in 40 mM Tris-HCl pH 7.4, 10 mM NaCl, 6 mM MgCl2, 10 mM CaCl2) was necessary (Ye et al., 2007). Periodic Acid Schiff (PAS) staining was carried out according to the manufacturer's instructions (Sigma). For quantifications, co-immunofluorescence was determined using the Analyze Particles function of ImageJ (NIH) and confirmed by eye in Adobe Photoshop. Calculations and graphs were generated with Microsoft Excel and R (www.r-project.org). P-values were determined by Tukey's HSD test in R and data are represented as mean +/- SEM. The numbers of mice used for each experiment are indicated in each graph. Acinar dissociation was performed as previously described (Kopinke and Murtaugh, 2010).

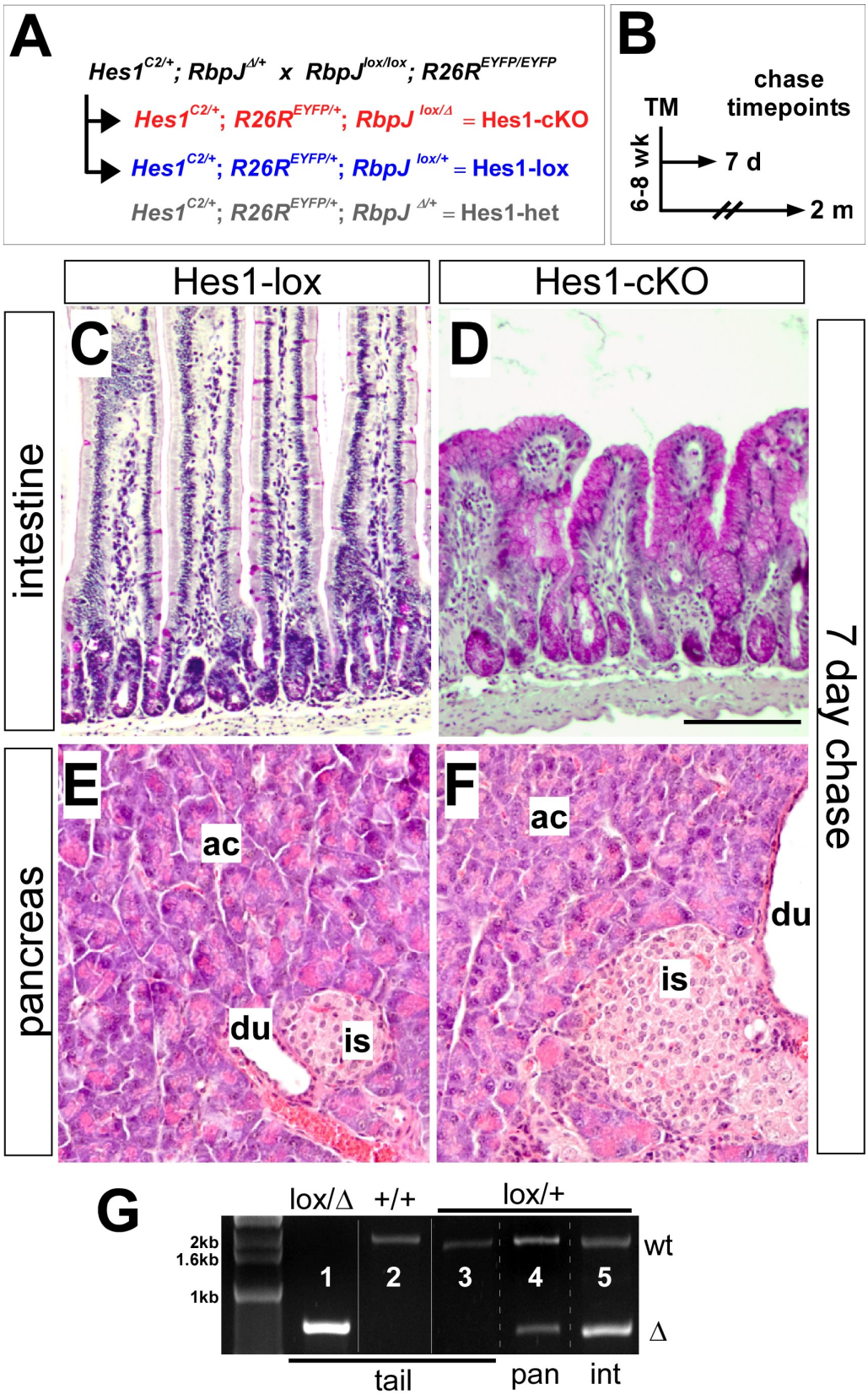
Results

Hes1-specific deletion of *RbpJ* in adult intestine and pancreas

RbpJ encodes the CSL transcription factor through which Notch activates target genes (Kopan and Ilagan, 2009). To determine a potential role for Notch signaling in rare $Hes1^{+}$ cells of the adult pancreas, we conditionally deleted a floxed *RbpJ* allele (Han et al., 2002) using our tamoxifen (TM)-inducible $Hes1^{CreERT2}$ line ($Hes1^{C2}$) (Kopinke et al., 2011). As depicted in Fig. 6.1A, our breeding scheme yielded both $Hes1^{C2/+}$; $R26R^{EYFP/+}$; $RbpJ^{lox/+}$ mice, which are heterozygous for the floxed allele (henceforth referred to as Hes1-lox), and $Hes1^{C2/+}$; $R26R^{EYFP/+}$; $RbpJ^{lox/\Delta}$ animals, which carry a null (Δ) and a floxed allele of *RbpJ* (Hes1-cKO). All genotypes also included the $R26R^{EYFP}$ reporter allele (Srinivas et al., 2001), which allowed us to follow the fate of recombined Hes1-lox and Hes1-cKO cells (see below). Hes1-cKO mice were born at normal Mendelian ratios and were phenotypically indistinguishable from wild-type or Hes1-lox animals before TM administration. It should be noted, however, that Hes1-cKO animals are compound heterozygotes for two major Notch components, *Hes1* and *RbpJ*. To exclude any defects caused by potential compound haploinsufficiency, we generated additional control mice that carry the null rather than the floxed allele of *RbpJ* ($Hes1^{C2/+}$; $R26R^{EYFP/+}$; $RbpJ^{\Delta/+}$; referred to as Hes1-het). In all experiments, unless otherwise indicated, 10 mg TM was administered to 6-8 week old adult mice, which were chased for 7 days (short term) or 2 months (long term) (Fig. 6.1B).

To confirm the successful deletion of *RbpJ* with our pulse-chase strategy, we first analyzed the small intestine of Hes1-lox and Hes1-cKO mice at 7 days post-TM. It was previously shown that pharmacological or genetic inhibition of Notch results in

Figure 6.1. Hes1-specific deletion of *RbpJ* in the pancreas and intestine. (A) Breeding strategy to generate a Hes1-specific knockout of *RbpJ*. Animals heterozygous for a null (Δ) and a floxed (lox) allele of *RbpJ* (*RbpJ*$^{lox/\Delta}$) are abbreviated Hes1-cKO, while *RbpJ*$^{lox/+}$ mice serve as controls (Hes1-lox). In some experiments, *RbpJ*$^{\Delta/+}$ mice were included as an additional control (Hes1-het). All mice also carry a *R26R*^{EYFP} reporter allele, allowing for lineage tracing of recombined cells. **(B)** Pulse-chase strategy. Recombination was induced by TM administration in 6-8 week old adults and animals were chased for 7 days (short term) or 2 months (long term) before analyzing. **(C-F)** Comparison of PAS-stained intestine (C-D) and H&E-stained pancreata (E-F) between Hes1-lox (C and E) and Hes1-cKO (D and F) mice after a 7 day chase. Intestinal KO of *RbpJ* (C) leads to widespread transformation of the gut epithelium into PAS^{+} goblet cells. In contrast, no morphological differences were detected between Hes1-lox (E) and Hes1-cKO (F) pancreata. **(G)** PCR strategy to detect successful recombination of the floxed allele of *RbpJ*. While the deletion band of *RbpJ* can only be detected in tail DNA from *RbpJ*$^{lox/\Delta}$ (1) but not wild-type (*RbpJ*$^{+/+}$, 2) or *RbpJ*$^{lox/+}$ (3) mice prior to TM, the band is visible post-TM-induced recombination using DNA from the pancreas (4) or duodenum (5) of the same *RbpJ*$^{lox/+}$ mouse (3). Abbreviations: ac, acinar; is, islet; du, duct. Scale bar: 100 μm.

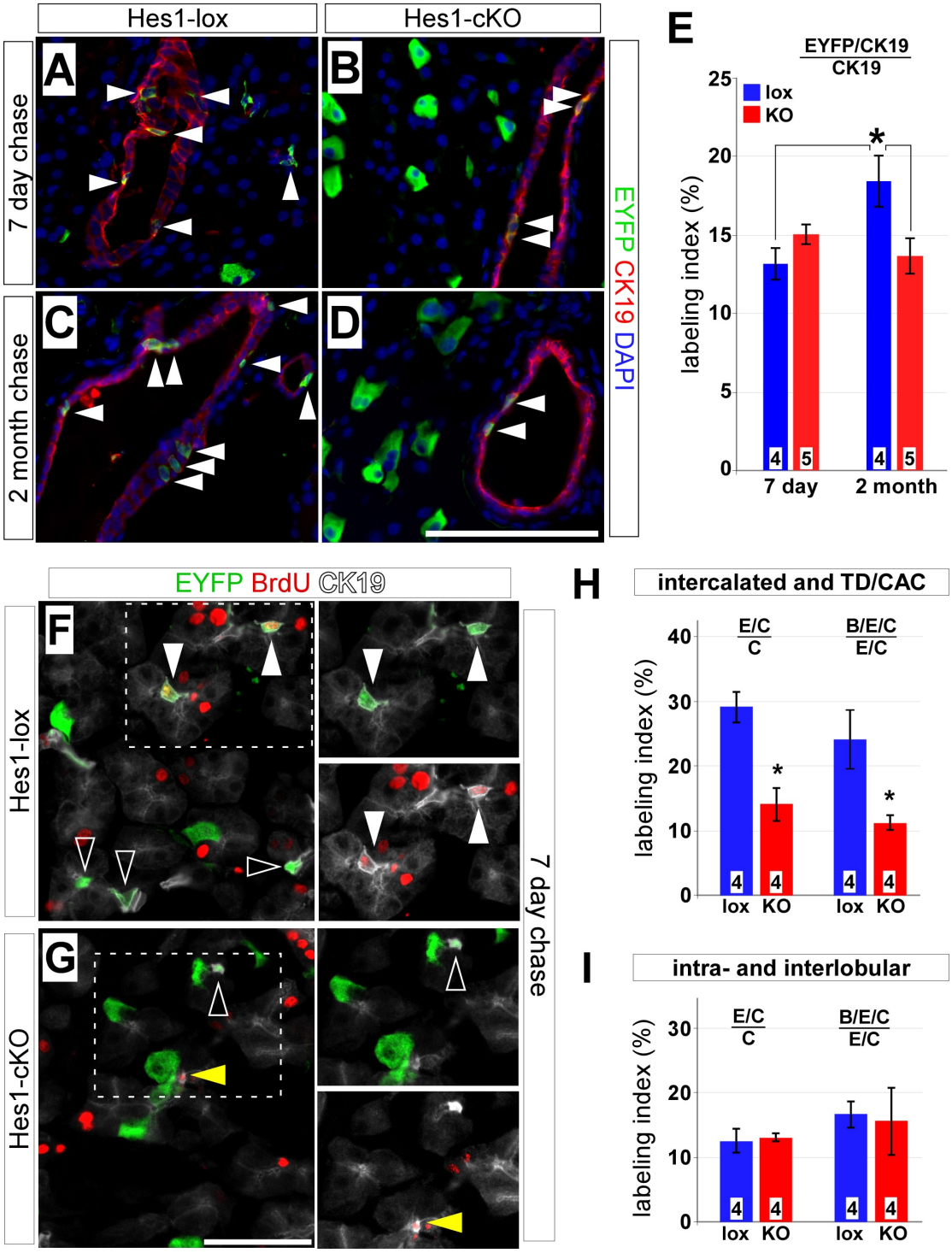

A
Hes1C2/+; RbpJΔ/+ x RbpJlox/lox; R26REYFP/EYFP
Hes1C2/+; R26REYFP/+; RbpJ lox/Δ = Hes1-cKO
Hes1C2/+; R26REYFP/+; RbpJ lox/+ = Hes1-lox
Hes1C2/+; R26REYFP/+; RbpJ Δ/+ = Hes1-het
B
TM
chase timepoints
6-8 wk
7 d
2 m
Hes1-lox
Hes1-cKO
intestine
pancreas
7 day chase
C
D
E
F
ac
du
is
G
lox/Δ
+/+
lox/+
2kb
1.6kb
1kb
1
2
3
4
5
wt
Δ
tail
pan
int

conversion of the intestinal epithelium into goblet cells (Kim and Shivdasani, 2011; Riccio et al., 2008; van Es et al., 2005). *Hes1*^{C2} labels intestinal stem cells (Kopinke et al., 2011), and deletion of *RbpJ* in the *Hes1*$^{+}$ stem cell compartment caused robust transformation of the gut epithelium into PAS^{+} positive goblet cells (Fig. 6.1C-D). *Hes1*-specific deletion of *RbpJ* in the pancreas, however, did not result in any obvious morphological differences between control and Hes1-cKO mice at 7 days post-TM (Fig. 6.1C-D). To confirm successful recombination in the pancreas, we performed PCR to detect the deletion (Δ) allele of *RbpJ* (Fig. 6.1G). As expected, the deletion-specific product could only be detected in the pancreas and intestine of Hes1-lox (lox/+) mice after TM-induced recombination of the floxed allele.

Deletion of *RbpJ* in *Hes1*-expressing duct cells blocks their expansion

We previously showed that *Hes1*^{C2} marks a subset of cells within large ducts of the adult pancreas, as well as CACs (Kopinke et al., 2011). Importantly, the main duct cells labeled by *Hes1*^{C2} appear to expand preferentially compared to unlabeled cells, suggesting that *Hes1* marks a proliferating subpopulation within the ducts. As a recent study suggested that Jagged1-Notch signaling was mitogenic for ducts (Golson et al., 2009), we analyzed *RbpJ* knockouts for any defects of the ductal tree. Since all mice also carry the Cre-dependent *R26R*^{EYFP} reporter allele, TM-induced recombination will result in simultaneous deletion of the floxed allele of *RbpJ* and activation of EYFP expression. This should allow us to directly and quantitatively compare Hes1-lox to Hes1-cKO cells by virtue of EYFP expression, and determine whether there was a change in the EYFP labeling frequency of ducts.

Figure 6.2. Deletion of *RbpJ* inhibits expansion of *Hes1*^{C2}-labeled ducts. **(A-D)** Adult Hes1-lox and Hes1-cKO mice were treated with tamoxifen and stained for EYFP (green) and the duct marker CK19 (red) after 7 days (A-B) and 2 months (C-D). While there is no difference in the fraction of EYFP-labeled duct cells (arrowheads) between 7 day chased Hes1-lox and Hes1-cKO animals (A-B), an increase is detected between 7 days and 2 month in Hes1-lox mice (C), which is inhibited in Hes1-cKO animals (D). **(E)** Quantification of duct EYFP labeling index. (**P*<0.05). **(F-G)** EYFP (green) and BrdU (red) labeling of CK19^{+} CACs (white), 7 days post-TM and following a 10 day BrdU pulse. CACs expressing EYFP only (open arrowheads) or positive for both EYFP and BrdU (closed arrowheads) can be found in Hes1-lox mice. In contrast, Hes1-cKO animals have fewer EYFP^{+} CACs, and most of the BrdU^{+} CACs are EYFP-negative (yellow arrowhead). **(H)** Quantifications of EYFP and BrdU labeling indices of intercalated ducts and CACs reveal a 2-fold decrease in EYFP labeling in Hes1-cKO mice, as well as a 2-fold reduction in the number of cycling (BrdU^{+}) EYFP-labeled intercalated ducts and CACs (* *P*<0.05). **(I)** Quantifications of EYFP and BrdU labeling indices of intra- and interlobular ducts indicate no differences between Hes1-lox and Hes1-cKO mice (*P*=0.75). Data are represented as mean +/- SEM. Numbers in bars (E, H, I) indicated mice analyzed per group. Scale bars: A-D and J, 100 μm; F-G, 50 μm.


Hes1-lox
Hes1-cKO
7 day chase
2 month chase
A
B
C
D
EYFP CK19 DAPI
E
EYFP/CK19
CK19
lox
KO
labeling index (%)
25
20
15
10
5
0
4
5
4
5
7 day
2 month
EYFP BrdU CK19
F
G
Hes1-lox
Hes1-cKO
7 day chase
H
intercalated and TD/CAC
E/C
C
B/E/C
E/C
labeling index (%)
40
30
20
10
0
4
4
4
4
lox
KO
lox
KO
I
intra- and interlobular
E/C
C
B/E/C
E/C
labeling index (%)
30
20
10
0
4
4
4
4
lox
KO
lox
KO

As previously (Kopinke et al., 2011), we could detect an increase in the fraction of labeled CK19$^+$ duct cells between 7 days and 2 months in Hes1-lox mice. By comparison, the labeling index of Hes1-cKO ducts remains the same regardless of the chase period (Fig. 6.2A-E) suggesting that the expansion of *Hes1*$^+$ ducts requires Notch activity. The ductal tree can be divided into large (intra- and interlobular) and small ducts (intercalated, terminal ducts and CACs) (Kopinke and Murtaugh, 2010). Since *Hes1*$^+$ cells are distributed throughout the ductal network, it is possible that loss of *RbpJ* results in different outcomes at different positions within the network. Therefore, Hes1-lox and Hes1-cKO ductal trees were analyzed for the contribution of EYFP$^+$ cells specifically to large and small ducts 7 days post-TM. Since terminal ducts and CACs are phenotypically similar and may represent one, two or more specific cell types (Ekholm et al., 1962; Rovira et al., 2010), we will henceforth refer to them collectively as CACs. Interestingly, the fraction of EYFP$^+$ intra- and interlobular ducts and their ability to proliferate did not change after a short-term loss of *RbpJ* (Fig. 6.2I). In contrast, we found an approximately 2-fold reduction in the fraction of EYFP$^+$ intercalated ducts and CACs, 7 days post-TM treatment. This decrease in labeled CAC was concomitant with reduced proliferation of these cells, evidenced by BrdU uptake and Ki67 staining (Fig. 6.2F-H and data not shown). Staining for cleaved Caspase-3 after a 2- or 7-day chase did not reveal increased apoptosis of *RbpJ*-deficient CACs (n=2 per genotype and time point, data not shown). Together, our data suggest that Notch signaling is required for the gradual expansion of *Hes1*$^+$ cells within large ducts, while the maintenance of *Hes1*$^+$ cells in intercalated ducts and CACs is acutely Notch-dependent. The apparent lack of cell death in the latter population raises the possibility that these cells change their

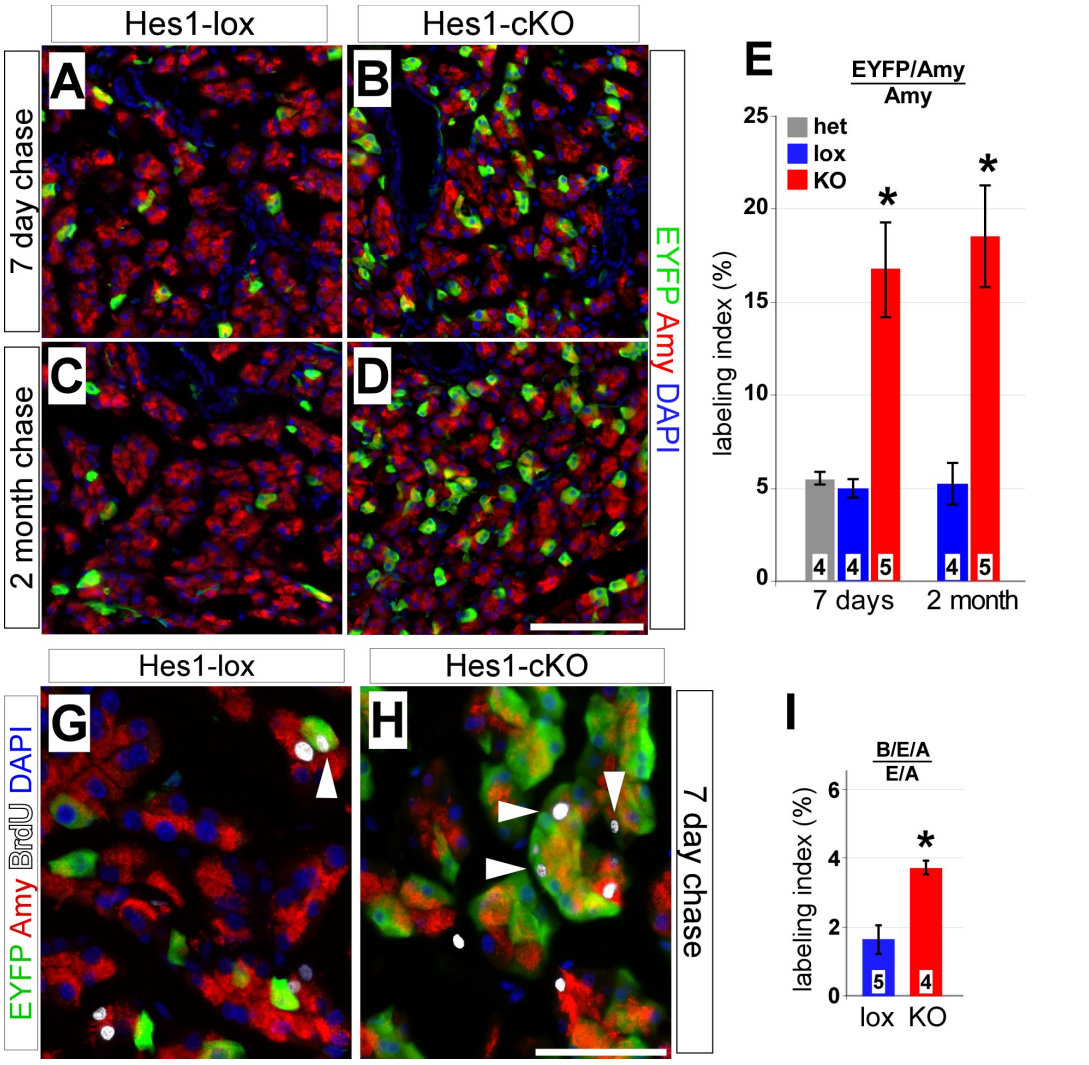


Figure 6.3. Loss of *RbpJ* in *Hes1*$^+$ cells results in dramatic increase of labeled acinar cells. **(A-D)** Adult Hes1-lox and Hes1-cKO pancreata were analyzed for co-expression of EYFP (green) with the acinar marker amylase (red), 7 days (A-B) and 2 months (C-D) post-TM. The fraction of EYFP$^+$ acini in Hes1-lox animals remains constant between 7 days (A) and 2 month (C). In contrast, a drastic increase in labeled acinar cells is seen both 7 days (B) and 2 month (D) after loss of *RbpJ*. **(E)** Quantification of the fraction of EYFP$^+$ acinar cells after a 7 day and 2 month chase. The fraction of EYFP-expressing acinar cells increases 3.5-fold at the 7 day and 2 month chase time points in Hes1-cKO pancreata (* P<0.005). **(G-H)** BrdU/EYFP labeling at 7 days post-TM. Only a few EYFP-expressing acinar cells are also positive for BrdU (white, arrowheads) in Hes1-lox mice (G). In contrast, Hes1-cKO animals display an increase in cycling EYFP$^+$ acinar cells (H). **(I)** Quantification of EYFP/BrdU labeling of acinar cells reveals a 2-fold increase in Hes1-cKO mice (* P<0.005). Data are represented as mean +/- SEM. Numbers in bars (E, I) indicated mice analyzed per group. Scale bars: A-D, 100 μm; G-H, 50 μm.

differentiated fate and, therefore, carry the EYFP label into a different lineage.

Dramatic increase of *Hes1*$^+$ acinar cells after loss of *RbpJ*

While analyzing the EYFP distribution within ducts, we were surprised to find a greater fraction of labeled acinar cells in Hes1-cKO pancreata compared to Hes1-lox (Fig. 6.3A-D). This effect was quite rapid: within 7 days of tamoxifen administration, Hes1-cKO pancreata exhibited an approximately 3.5-fold increase in labeled acinar cells, which did not increase further after 2 months (Fig. 6.3E). This increase in labeling frequency was not due to compound haploinsufficiency for *RbpJ* and *Hes1*, as the labeling index of Hes1-het mice was indistinguishable from Hes1-lox. Notch signaling has previously been suggested to inhibit acinar cell proliferation (Siveke et al., 2008). To determine whether the expanded acinar labeling in Hes1-cKO could be attributed entirely to division of rare *Hes1*^{C2}-labeled acinar cells (Kopinke et al., 2011), we continuously supplied Hes1-lox and Hes1-cKO mice with BrdU in the drinking water from 3 days prior to TM through harvesting, in order to capture all cycling cells during our chase period (Teta et al., 2007). After a 7 day chase, ~2% of EYFP$^+$ acinar cells are positive for BrdU in Hes1-lox mice, compared to ~4% in Hes1-cKO mice (Fig. 6.3G-I). Because the initial fraction of BrdU$^+$ acinar cells is very low, and increases only 2-fold in Hes1-cKO mice, accelerated proliferation and expansion of *Hes1*$^+$ acinar cells upon loss of *RbpJ* seems unlikely to explain fully the dramatic increase in EYFP$^+$ acinar cells.

To test more directly the effects of *RbpJ* on acinar cell proliferation, we sought to delete this gene within this compartment specifically. For this, we used a TM-inducible

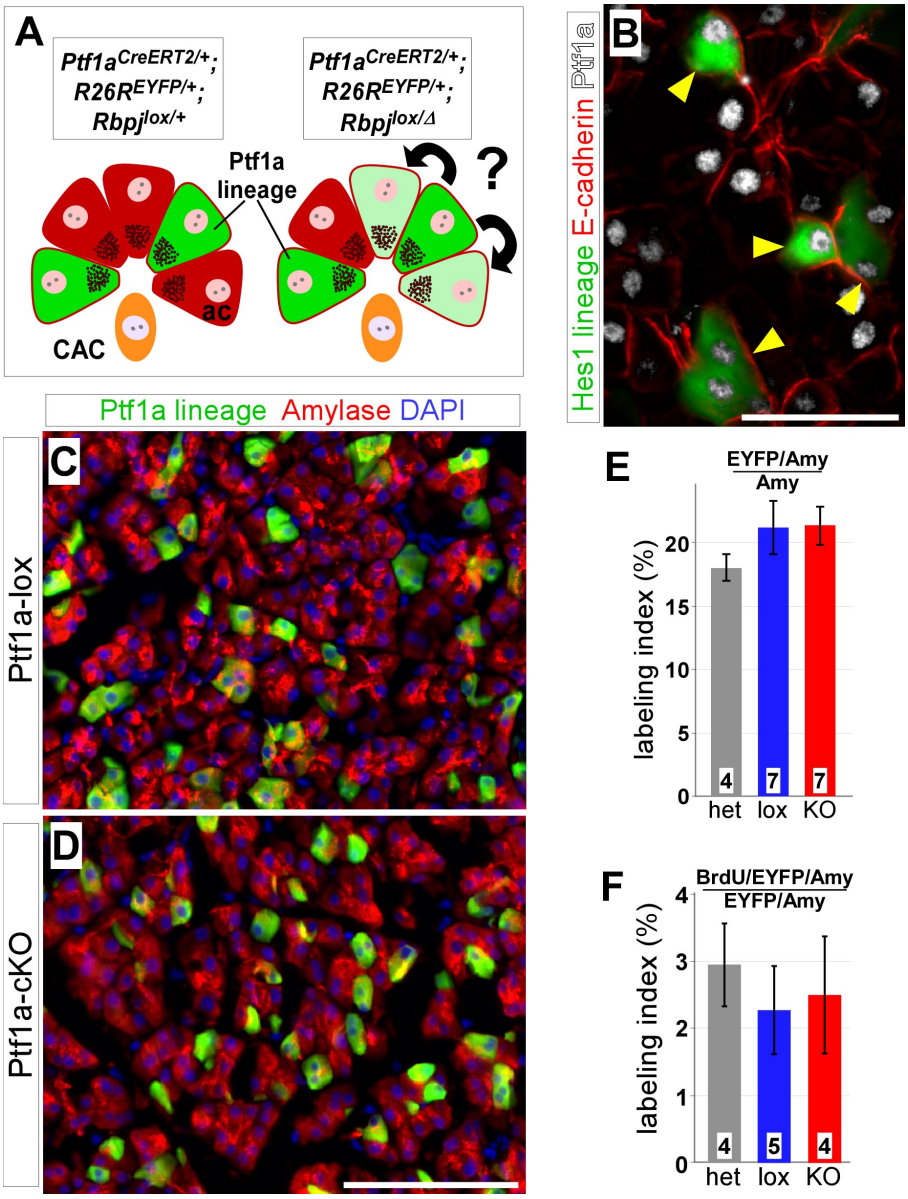


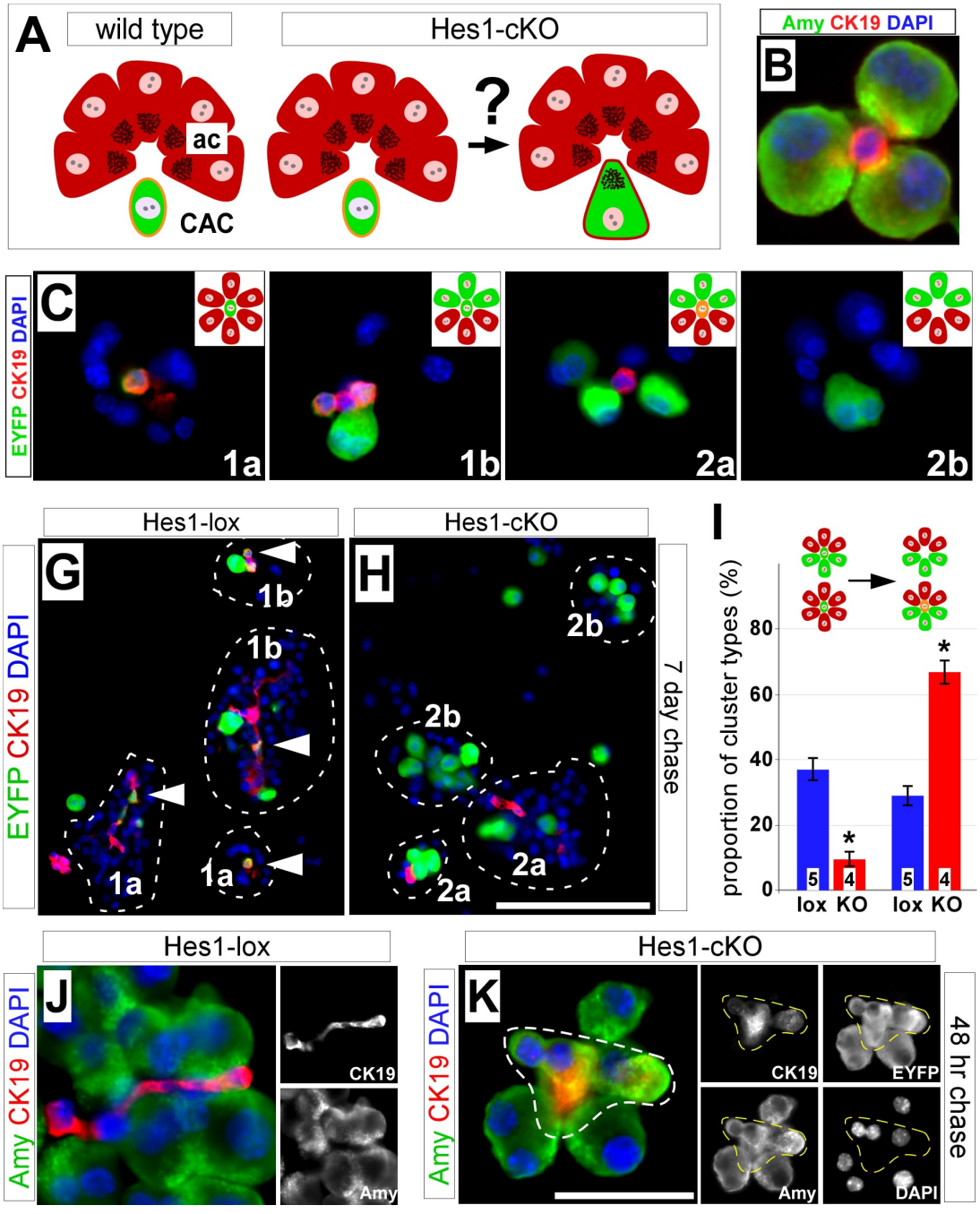
Figure 6.4. No increased proliferation after acinar-specific loss of *RbpJ*. **(A)** To determine whether Notch generally represses expansion of acinar cells, *RbpJ* was deleted using an inducible *Ptf1a*^{CreERT} Cre driver, which induces mosaic recombination in acinar cells (Ptf1a-lineage, green) but not ducts or CACs. **(B)** Short term lineage tracing of *Hes1*$^{+}$ cells (green) demonstrates that all EYFP^{+} acinar cells (arrowheads) also express Ptf1a (white). **(C-D)** At 7 days post-TM, no difference is detected, between Ptf1a-lox and Ptf1a-cKO, in EYFP labeling (green) of amylase^{+} acinar cells (red). **(E)** There is no change in the fraction of EYFP^{+} acinar cells after a 7 day chase (P=0.52). **(F)** Quantification of BrdU labeling analysis 7 days post-TM. The EYFP/BrdU labeling index of acinar cells remains the same between control (lox and het) and Ptf1a1-KO animals (P=0.81). Data are represented as mean +/- SEM. Numbers in bars (E-F) indicate mice analyzed per group. Scale bars: B, 50 μm; C-D, 100 μm.

Cre line under the control of the acinar-specific transcription factor Ptf1a (*Ptf1a*^{CreERT}) (Fig. 6.4A). Immunostaining confirmed that Ptf1a was expressed by all acinar cells, including the subpopulation labeled by *Hes1*^{C2} (Fig 6.4B). Similar to our *Hes1*^{C2} breeding scheme (Fig. 6.1A), we generated mice containing floxed and wild-type *RbpJ* alleles (Ptf1a-lox, *Ptf1a*$^{CreERT/+}$; *R26R*$^{EYFP/+}$; *RbpJ*$^{lox/+}$), or a floxed and null (Ptf1a-cKO, *Ptf1a*$^{CreERT/+}$; *R26R*$^{EYFP/+}$; *RbpJ*$^{lox/\Delta}$). As before, all mice also carried one allele of the *R26R*^{EYFP} reporter for lineage tracing (Fig. 6.4A). We found no difference between Ptf1a-lox and Ptf1a-cKO in the EYFP labeling of acinar cells at 7 days post-TM (Fig. 6.4C-E). Using the same BrdU labeling scheme as above, we also detected no change in the fraction of EYFP-expressing, BrdU$^+$ acinar cells between genotypes (Fig. 6.4F). These results argue against a role for Notch in regulating proliferation/expansion of mature acinar cells, and raise the possibility of a Notch-regulated influx to the acinar compartment from another cell type.

Loss of *RbpJ* causes rapid transdifferentiation of CAC into acinar cells

The disappearance of EYFP$^+$ intercalated ducts and CACs (Fig. 6.2), and our finding that Notch does not inhibit acinar proliferation (Fig. 6.4), prompted us to investigate whether the observed increase of EYFP$^+$ acinar cells in Hes1-cKO mice was due to transdifferentiation of CACs (Fig. 6.5A). To analyze individual acinar units, and avoid missing small CACs due to sectioning artifacts, we performed enzymatic digestion to dissociate the pancreas into clusters containing only acinar cells and CACs (referred to as acinar preps; Fig. 6.5B) (Kopinke and Murtaugh, 2010; Kurup and Bhonde, 2002). Using this method, we scored four different cluster types, which we divided into two

Figure 6.5. Transdifferentiation of *Hes1*$^+$ CAC into acinar cells upon *RbpJ* knockout. **(A)** We hypothesize that the increase in acinar labeling after loss of *RbpJ* is due to CACs adopting an acinar fate. **(B)** Dissociation of whole pancreata generates small cell clusters containing amylase$^+$ acinar cells (green) and CK19$^+$ CACs (red). **(C)** Clusters from Hes1 lineage traced (green) pancreata were divided into two major categories, based on the presence (class 1) or absence (class 2) of labeled CACs as indicated. Class 1 clusters contain only EYFP$^+$ CACs (1a) or EYFP$^+$ CACs and acini (1b) and class 2 clusters contain EYFP$^+$ acini with unlabeled CACs (2a) or EYFP$^+$ acini with no CACs (2b). Inserts represent schematic representations of cluster types. **(G-H)** Immunofluorescence for EYFP (green) and CK19 (red) of clusters from dissociated pancreata of Hes1-lox and Hes1-cKO animals at 7 days post-TM. Only clusters containing 3 or more acinar cells were scored (circle). Most of the Hes1-lox clusters contain labeled CAC (arrowhead) and belong to class 1, while CACs are either unlabeled or absent altogether in Hes1-cKO clusters (classes 2a and 2b). **(I)** Scoring of cluster types from two independent experiments demonstrates a shift in the proportion of clusters from type 1a and 1b to 2a and 2b between Hes1-lox and Hes1-cKO mice (* P<0.0005). Note that the denominator represents the number of total clusters present per field, including EYFP-negative ones. **(J-K)** Immunofluorescence for amylase (green) and CK19 (red) of cell clusters from Hes1-lox (J) and Hes1-cKO (K) pancreata 48 hrs after TM administration. In Hes1-lox mice (J), CK19 and amylase expression are restricted to CAC and acinar cells, respectively. After TM induction (K), some amylase$^+$ acinar cells are also positive for CK19 (dotted outline). Co-expression of duct and acinar markers is seen only in EYFP$^+$ cells (right). Right, single-channel amylase, CK19, EYFP and DAPI staining as indicated. Data are represented as mean +/- SEM. Numbers in bars (I) indicate mice analyzed per group. Scale bar: G-H, 50 μm; J-K, 25 μm.


A
wild type
Hes1-cKO
ac
CAC
?
B
Amy CK19 DAPI
C
EYFP CK19 DAPI
1a
1b
2a
2b
Hes1-lox
Hes1-cKO
G
H
7 day chase
I
proportion of cluster types (%)
lox KO lox KO
J
K
Amy CK19 DAPI
CK19
Amy
EYFP
DAPI
48 hr chase

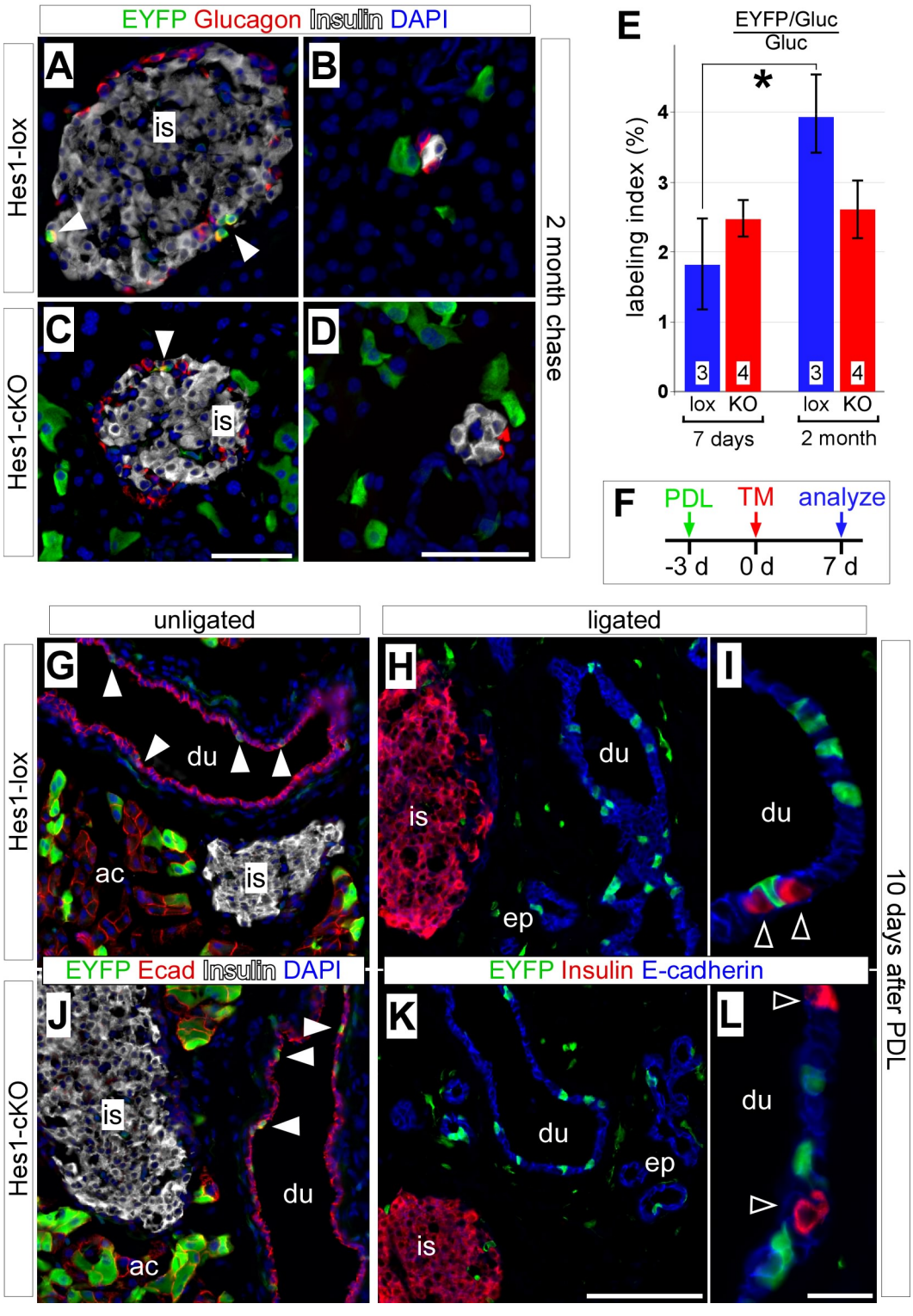
major categories based on the presence (class 1) or absence (class 2) of EYFP-labeled CACs. In class 1, we scored clusters in which only CACs were labeled (1a) or in which both CACs and acini were labeled (1b). In class 2, we scored clusters containing labeled acini with unlabeled CACs (2a) or labeled acini with no CACs at all (2b). If CACs were converting to acinar cells after *RbpJ* deletion, we would expect a decrease in class 1 clusters and an increase in class 2. Indeed, we found that the majority of Hes1-lox clusters were of class 1, while class 2 predominated in Hes1-cKO (Fig. 6.5G-H). Quantification revealed a 3.5-fold reduction, in Hes1-cKO mice, of the class 1 cluster frequency, and a concomitant increase (2.5-fold) of class 2 clusters (Fig. 6.5I), suggesting that CACs convert to acinar cells after loss of *RbpJ*.

If CACs are indeed capable of adopting an acinar fate, we should detect transitional cells expressing both duct and acinar markers. By analyzing acinar preps from a 48 hr chase, we were able to detect $EYFP^+$ cells co-expressing the duct marker CK19 and the mature acinar marker amylase in Hes1-cKO mice specifically (Fig. 6.5J-K). At later chase time points, we no longer observed EYFP-labeled double-positive cells. Thus, loss of *RbpJ* in CACs causes a rapid and complete transition to an acinar fate. These results suggest that *Hes1*$^+$ CACs can act as facultative acinar progenitors, and that their differentiation status is controlled by Notch activity.

Lack of exocrine-to-endocrine conversion after *RbpJ* deletion

Wild-type *Hes1*$^+$ cells, including CACs, do not contribute to new β-cells during normal homeostasis or after PDL (Kopinke et al., 2011). Nonetheless, the results above indicate that loss of *RbpJ* allows CACs to adopt a cell fate from which they are normally

Figure 6.6. Loss of *RbpJ* does not cause exocrine to endocrine conversion. **(A-D)** Adult Hes1-lox and Hes1-cKO pancreata were analyzed for expression of EYFP (green) by glucagon$^+$ α-cells (red) and insulin$^+$ β-cells (white), at 2 months post-TM. While fewer α-cells are marked by EYFP (arrowhead) in Hes1-cKO (C) compared to Hes1-lox animals (A), no β-cells are labeled either in large islets (A and C) or in small clusters (B and D), regardless of genotype. **(E)** Quantification of EYFP/glucagon labeling 7 days and 2 month post-TM. The expansion of EYFP-labeled α-cells between 7 days and 2 month, seen in Hes1-lox animals (* P<0.05), is blocked in Hes1-cKO mice. **(F)** Adult Hes1-lox and Hes1-cKO animals were subjected to PDL, received TM three days after and were chased for 7 additional days before analyzing. **(G-L)** Comparison of unligated (G and J) and ligated areas (H-I and K-L) from Hes1-lox (G-I) and Hes1-cKO pancreata (J-L) 10 days after PDL. Hes1-lox (G) and Hes1-cKO (J) EYFP-expressing cells (green) can be found within ducts (du, closed arrowheads) and acinar cells (ac) in the unligated pancreas but not in β-cells (white) of islets (is). In ligated areas of both genotypes, EYFP$^+$ cells are present only in ducts and epithelial clusters (ep, marked by E-cadherin in blue) (H and K). Individual unlabeled β-cells (red, open arrowheads) can be found in proximity to EYFP$^+$ ducts in Hes1-lox (I) and Hes1-cKO mice (L). Data are represented as mean +/- SEM. Numbers in bars (E) indicate mice analyzed per group. Scale bars: A-D, G-H and J-K 100 μm; I and J, 10 μm.


EYFP Glucagon Insulin DAPI
Hes1-lox
Hes1-cKO
2 month chase
A
B
C
D
is
E
EYFP/Gluc
Gluc
labeling index (%)
lox KO
7 days
2 month
F
PDL
TM
analyze
-3 d
0 d
7 d
unligated
ligated
G
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ep
EYFP Ecad Insulin DAPI
EYFP Insulin E-cadherin
10 days after PDL

excluded. We therefore wanted to determine whether loss of *RPB-J* in *Hes1*$^+$ cells could allow them to adopt a β-cell fate as was shown in vitro (Rovira et al., 2010). We first looked for a possible contribution to new β-cells during normal homeostasis, and could not find a single insulin$^+$ β-cell labeled by EYFP after a 7-day or 2-month chase in Hes1-cKO mice (Fig. 6.6A-D). We did find, however, that the progressive expansion of EYFP-labeled α-cells (glucagon$^+$), observed in Hes1-lox and wild-type mice (Kopinke et al., 2011), was abolished in Hes1-cKO animals (Fig. 6.6A-E). Although these cells are a minute fraction of the total α-cell population, our results suggest that their expansion, like that of *Hes1*$^+$ duct cells, is Notch-dependent.

It has been suggested that duct cells can generate new β-cells under various injury settings, including pancreatic duct ligation (PDL) (Murtaugh and Kopinke, 2008; Pan and Wright, 2011). In this model, re-expression of the pro-endocrine transcription factor Neurog3 can be observed within ductal structures (Xu et al., 2008). Since Hes1 represses the *Neurog3* promoter (Lee et al., 2001), we wanted to determine whether loss of *RbpJ* in Hes1-expressing duct cells would enhance *Neurog3*-dependent β-cell neogenesis to the point that it could be directly observed by lineage tracing. Hes1-lox and Hes1-cKO mice received TM 3 days after duct ligation and were chased for 7 additional days. In the ligated areas of Hes1-lox and Hes1-cKO mice around 15% and 22% of CK19$^+$ ducts were labeled, respectively (Fig. 6.6G-L). After scoring several thousand insulin$^+$ β-cells and EYFP$^+$ ducts, however, we did not detect any β-cells expressing EYFP (Table S1). Thus, *Hes1*$^+$ duct cells, even after losing *RbpJ*, are not capable of giving rise to new β-cells.

CAC contribute to new acinar cells following acute pancreatitis

Our finding that *RbpJ* deletion causes CAC-to-acinar transdifferentiation prompted us to determine whether wild-type CACs can also contribute to acinar cells under physiological conditions. Since no influx from CACs to acini was detected during steady state conditions (Kopinke et al., 2011; Kopp et al., 2011), we utilized an exocrine regeneration model in which acute pancreatitis is induced by supraphysiological levels of the acinar secretagogue caerulein (Jensen et al., 2005). This treatment paradigm causes widespread acinar cell death within 2-3 days of treatment, followed by complete recovery of acinar mass 7-14 days later (Jensen et al., 2005). The extensive acinar turnover in this model provided a system to investigate whether wild-type $Hes1^{+}$ CACs could contribute to newly-generated acinar cells. To this end, we induced acute pancreatitis in $Hes1^{C2/+}$; $R26R^{EYFP/+}$ mice and determined the fate of wild-type, $Hes1^{C2}$-labeled cells.

Measurement of serum amylase confirmed robust injury induced by caerulein treatment. At 14 days after the last caerulein injection, we found no gross or histological difference between saline- and caerulein-treated mice, demonstrating that the pancreas of treated animals had fully regenerated (Fig. 6.7A-B and data not shown). Next, we examined the distribution of EYFP-expressing cells at the 14-day timepoint. Saline-treated $Hes1^{C2/+}$; $R26R^{EYFP/+}$ mice exhibited a homogenous low frequency of EYFP-labeled acinar cells, similar to our previous findings (Kopinke et al., 2011). In contrast, caerulein treatment caused an apparent focal increase in EYFP-labeled acinar cells (Fig. 6.7A-B), indicating either that Hes1-derived acinar cells expanded, or that EYFP-marked CACs contributed to new acinar cells. The increased heterogeneity of labeling, following caerulein treatment, suggested that injury had affected some regions of the organ more

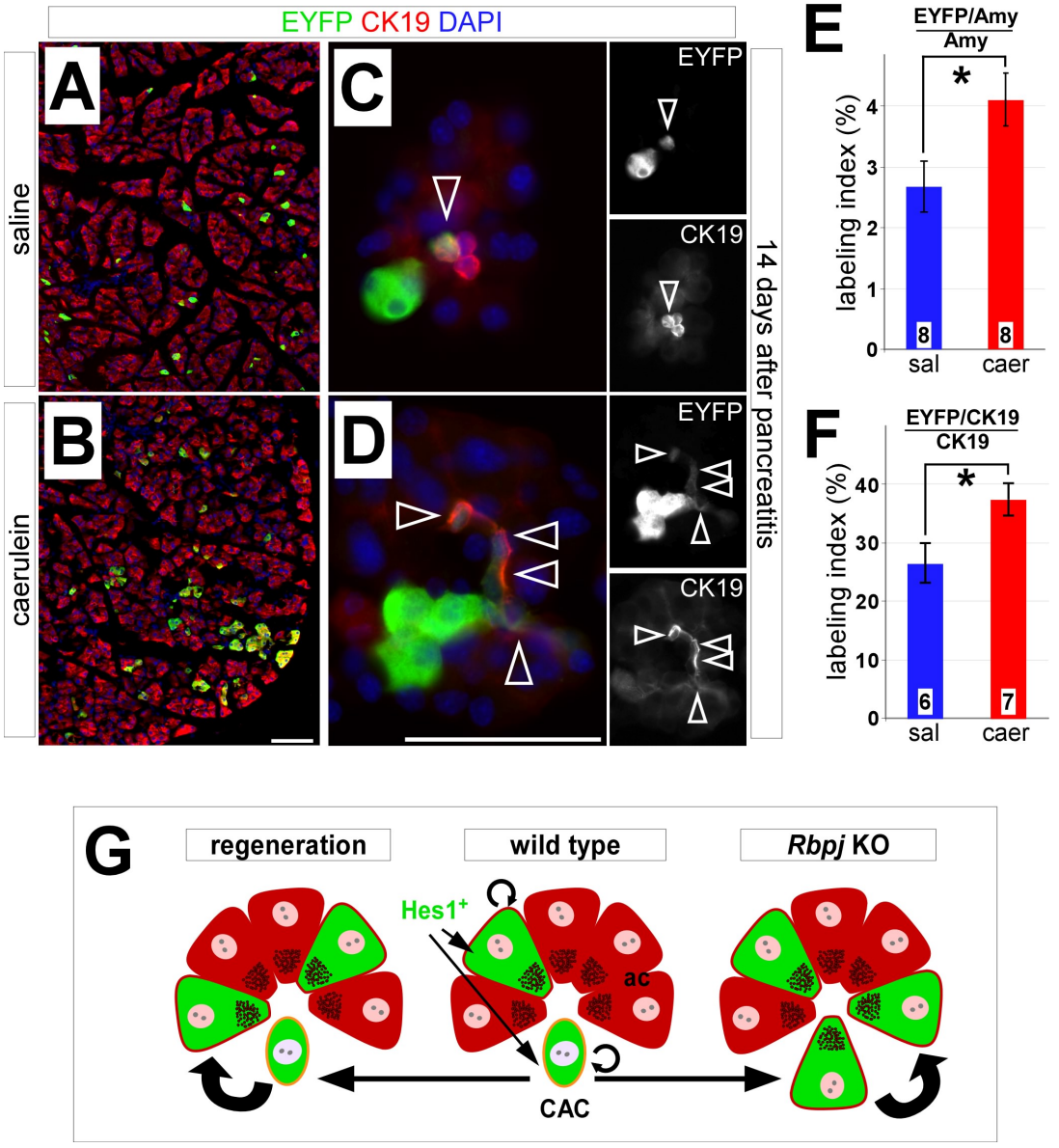

Figure 6.7. Contribution of *Hes1*$^{+}$ CAC to new acinar cells following pancreatitis. **(A-D)** Sections (A-B) and dissociated acinar clusters (C-D) from adult *Hes1*$^{CF/+}$; *R26R*$^{EYFP/+}$ pancreata were analyzed for EYFP (green) and CK19 (red) expression 14 days after caerulein-induced acute pancreatitis. In contrast to saline treated mice (A and C), caerulein treatment (B and D) causes an increase in the fraction of *Hes1*^{C2}-labeled acinar cells as well as marked CACs (arrowheads). **(E)** Quantifying the EYFP labeling index of acinar cells at 14 days post-caerulein reveals an increase in caerulein-treated mice (* $P<0.05$). **(F)** Scoring the fraction of labeled CK19^{+} cells indicates a slight increase in EYFP^{+} CAC after regeneration (* $P<0.05$). **(G)** Model: *Hes1*$^{+}$ CACs are maintained by Notch signaling and can generate new acinar cells during regeneration. Data are represented as mean +/- SEM. Numbers in bars (C, D) indicate mice analyzed per group. Scale bar: 50 μm.

than others, which could be over- or under-counted in sections. Therefore, we utilized our acinar prep technique for quantifications, allowing randomization of acinar clusters regardless of regional variation. The fraction of labeled acinar cells (Fig. 6.7C, E) and CACs (Fig. 6.7C, F) in saline-treated animals was similar to our previous data (Kopinke et al., 2011). In caerulein-treated mice, however, we observed an increase in the fraction of $EYFP^+$ acinar cells (Fig. 6.7D-E) as well as CACs (Fig. 6.7F), suggesting that the *Hes1*-expressing CACs expand and give rise to new acinar cells following pancreatitis. Together with our *RbpJ* deletion experiments, these results suggest that $Hes1^+$ CACs represent facultative, acinar-restricted progenitor cells in the adult pancreas (Fig. 6.7G).

Discussion

The mammalian pancreas is a generally static organ, and many studies support replication as the major mode of postnatal expansion, adult homeostasis and regeneration (Desai et al., 2007; Dor et al., 2004; Kopinke and Murtaugh, 2010; Kopp et al., 2011; Solar et al., 2009; Strobel et al., 2007; Teta et al., 2007). Nonetheless, adult cell fates can be reprogrammed by the ectopic activation of developmental regulatory factors (Collombat et al., 2009; De La O et al., 2008; Zhou et al., 2008). While such gain-of-function experiments reveal the potential of adult cells to change fates, they do not address the mechanisms by which cells normally maintain phenotypic fidelity. Here, we provide the first evidence that an endogenous signaling pathway acts to prevent cell type interconversion in the adult pancreas. Centroacinar cells can be distinguished from other cells of the ductal network by their high level of Notch pathway activity (Kopinke et al., 2011; Miyamoto et al., 2003; Parsons et al., 2009; Stanger et al., 2005), and we find that

this activity is required constitutively to prevent their differentiation into acinar cells. Although wild-type CACs are inhibited from differentiating into acinar cells in the resting pancreas, our studies further suggest that they can contribute to regenerating acinar cells after acute pancreatitis. In this respect, CACs behave as facultative stem cells of the exocrine pancreas.

Based on lineage tracing of pre-existing acinar cells, two previous studies had argued against a major contribution of nonacinar cells to the acinar compartment following pancreatitis (Desai et al., 2007; Strobel et al., 2007). These studies could not exclude a small contribution from nonacinar cells, however, particularly since they did not directly label CACs. In fact, it has been shown that CAC proliferate rapidly shortly after the acute injury phase of caerulein-induced pancreatitis (Elsasser et al., 1986; Gasslander et al., 1992; Rovira et al., 2010), and radionucleotide uptake studies have suggested that CACs might migrate into other pancreatic cell compartments during the regeneration phase (Gasslander et al., 1992). We find that regeneration induces both an expansion of *Hes1*^{C2}-labeled cells within the CAC compartment, and contribution of these cells to differentiated acini. The latter phenomenon accounts for only a small fraction of regenerated acinar mass, however, which could have been missed in prior studies (Desai et al., 2007; Strobel et al., 2007). It will be interesting to determine if regeneration from more extreme acinar injury evokes a stronger contribution from CACs, as predicted by studies of facultative stem cells in the liver (Yanger and Stanger, 2011).

The expansion of *Hes1*$^{+}$ CACs during regeneration may reflect a mitogenic role for the Notch signaling pathway, which is upregulated during pancreatitis (Jensen et al., 2005). We have previously found that *Hes1*$^{+}$ cells within the main ducts preferentially

expand over time (Kopinke et al., 2011), and we show here that this expansion requires *RbpJ*. These findings converge with a previous study in which loss of Jagged1, a Notch ligand, was shown to cause hypoplastic and malformed ducts (Golson et al., 2009). Although loss of *RbpJ* inhibits the progressive expansion of $EYFP^+$ cells within large ducts, it does not have any detectable short-term effect on their proliferation. Therefore, we propose that the direct requirement for *RbpJ* is manifested in CACs, where Notch signaling sustains a pool of cells that transition into the ductal tree as it gradually expands during postnatal organ growth. Rapid CAC-to-acinar differentiation after loss of *RbpJ* depletes this pool, preventing further contribution to large ducts.

Are *Hes1*-expressing CACs unique, or do they share properties with other ductal cells? In analyzing conditional *RbpJ* knockouts, we have not observed ectopic, EYFP-labeled acinar cells within large ducts, suggesting that cells in these structures are incapable of changing fate even after removal of Notch activity. Furthermore, CACs were recently shown to have a unique capacity for multilineage differentiation in cell culture, although the cells isolated in that study appeared to be *Hes1*-negative (Rovira et al., 2010). With respect to *in vivo* differentiation potential, two other genes expressed by ducts and CACs, *Hnf1b* and *Sox9*, have been used to generate tamoxifen-inducible Cre lines. Lineage tracing with a *Hnf1b-CreERT2* BAC transgene demonstrated that ducts and CACs did not contribute to other cell types during homeostasis or following duct ligation (Solar et al., 2009). Similar results were obtained with a *Sox9*-specific BAC transgene (*Sox9-CreERT2*), indicating that postnatal ducts and CACs do not contribute to endocrine or acinar cells (Kopp et al., 2011). Directly conflicting this finding, however, studies using another *Sox9* lineage tracing allele ($Sox9^{IRES\text{-}CreERT2}$) showed that the

majority of mature acinar cells were repopulated from *Sox9*-expressing ducts or CACs within 4 months (Furuyama et al., 2011). This report is difficult to reconcile with the "pulse-chase" acinar cell labeling experiments described above, which concluded that long-term maintenance of acinar cells was based on replication of pre-existing ones (Desai et al., 2007; Strobel et al., 2007). Pending resolution of the Sox9 lineage, the weight of the published evidence would suggest that neither ducts nor CACs behave as endocrine or exocrine stem cells *in vivo*, while our findings here suggest that *Hes1*$^{+}$ CACs represent a facultative progenitor population specific to acinar cells (Fig. 6.7G).

Previous studies of Hes1 expression during embryonic pancreas development indicated a progressive restriction to cells at the border between acini and ducts (Esni et al., 2004), which our lineage tracing experiments identified as an exocrine-specific progenitor population in which Notch promotes duct differentiation at the expense of acinar (Kopinke et al., 2011). The experiments reported here suggest that these progenitors persist into adulthood, within an anatomical niche ideally suited for bipotential lineage contribution. Located at the termini of the ductal tree, self-renewing CACs can transit into the central ductal network and contribute to its overall expansion. At the same time, sustained Notch signaling ensures that these cells are available for replacement of acinar cells lost to injury. Notch activity is required for the exocrine pancreas to regenerate from pancreatitis (Siveke et al., 2008), and our studies highlight the unique contribution of Notch-regulated cell fate determination to the maintenance of this organ. In addition, our findings raise the question of whether other pathways controlling pancreatic organogenesis continue to play analogous roles in postnatal life.

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CHAPTER 7

SUMMARY AND CONCLUSIONS

Loss of insulin-producing β-cells results in type 1 diabetes, which can be counterbalanced through the transplantation of new β-cells. At the onset of my studies, it was unknown whether the pancreas contains an endogenous stem/progenitor cell population or a differentiated cell type that has the potential of giving rise to new β-cells in vivo (neogenesis). This thesis addressed the following questions: (1) What is the origin of the new β-cells that arise in large numbers shortly after birth? (2) Is there any contribution to new islets from the exocrine tissue (duct and acinar cells) during adult homeostasis or regeneration? (3) Do *Hes1*-expressing cells constitute multipotent progenitors throughout pancreatic organogenesis? (4) Do the rare *Hes1*$^{+}$ cells in the mature pancreas represent adult progenitors similar to their embryonic counterparts?

The first part of this thesis focused on the embryonic origin of β-cells, through genetic lineage tracing with a novel *Muc1*^{CreERT2} allele that is expressed specifically in exocrine acinar and ductal cells. During embryogenesis, we could show that *Muc1*$^{+}$ ducts can differentiate into β-cells and other islets. Following birth, however, we found that *Muc1*$^{+}$ cells completely fail to contribute to new islet β-cells. Thus, our work proves the ductal origin of endocrine cells during embryogenesis and indicates that both the

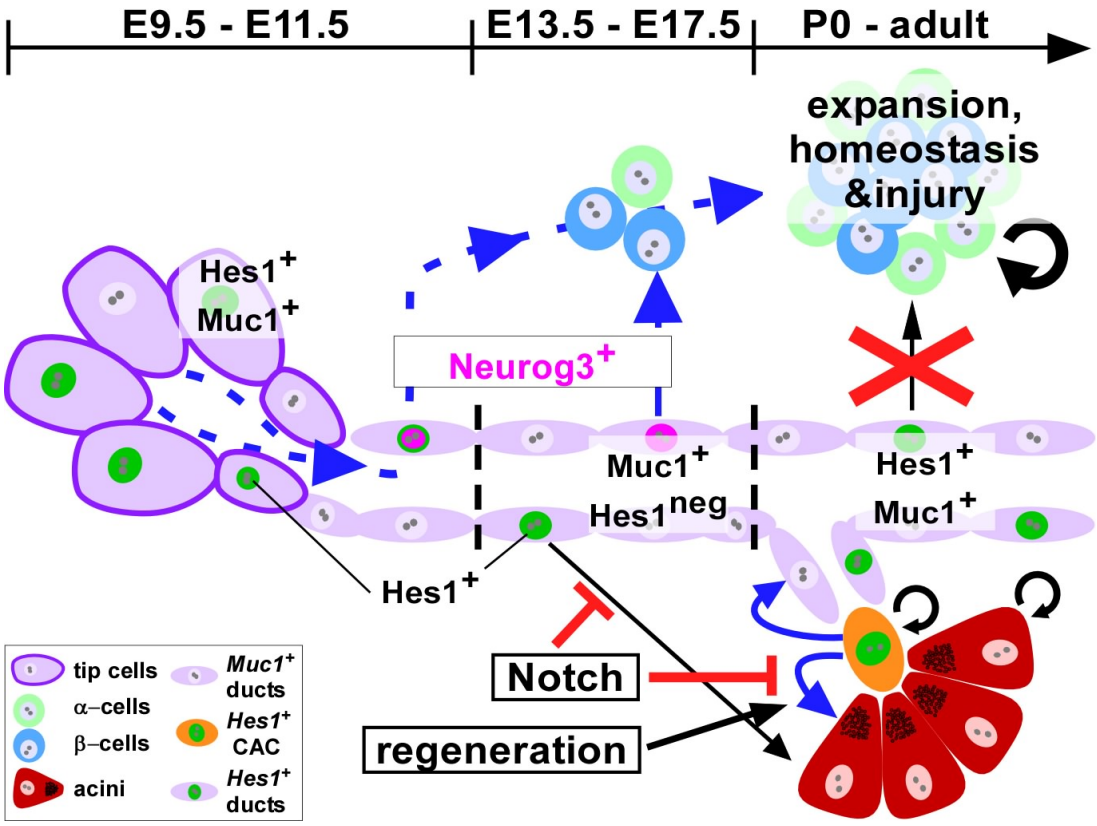

Figure 7.1. Model. Between E9.5 and E11.5, *Hes1*$^+$ and *Muc1*$^+$ cells are multipotent and give rise to islet cells through *Neurog3*-expressing intermediates. Starting at E13.5 until birth, endocrine cells arise from *Muc1*$^+$ ducts, which have downregulated Notch activity. *Hes1*$^+$ cells at E13.5 represent bipotent exocrine progenitors in which Notch inhibits acinar fate. After birth and in the adult, neogenesis from *Muc1*$^+$ and *Hes1*$^+$ ducts ceases and islet cells are maintained by proliferation of differentiated cells. *Hes1* expression persists in Notch-sensitive centroacinar cells, which act as facultative exocrine-specific progenitor cells capable of differentiating into duct (during homeostasis) and acinar cells (regeneration after pancreatitis).

expansion and homeostasis of adult islets occurs independent of contribution from ducts or acini (Chapter 3 and Fig. 7.1).

The second part investigated the possibility of ductal-to-islet differentiation during pancreatic ductal ligation (PDL) injury. This injury was shown to induce doubling of β-cell mass possibly caused by influx of new islet cells from ducts (Xu et al., 2008). Therefore, we performed lineage tracing during the regeneration process to elucidate whether new islet cells can arise from *Muc1*$^{+}$ ducts or acini. In fact, although we could detect robust labeling of duct-like cells, often in very close association with insulin^{+} cells, we failed to detect a single *Muc1*-derived β-cell after PDL. Our findings, therefore, argue against a major role for adult neogenesis even in the context of injury and regeneration (Chapter 4 and Fig. 7.1).

As duct cells have been proposed as a potential source for β-cell neogenesis, several duct-specific Cre lines have been developed since 2008 (Table 7.1). Three of these new Cre-drivers, *Hnf1bCreER* (Solar et al., 2009), *Sox9CreER* (Kopp et al., 2011) and *Sox9*$^{IRES\text{-}CreERT2}$ (Furuyama et al., 2011), have been used to perform lineage tracing after PDL. None of these studies, however, found signs of ducts giving rise to new β-cells. This raises the question whether neogenesis actually occurs after PDL. So far, three lines of evidence suggest neogenesis in this injury model: (i) β-cell mass undergoes doubling, (ii) *Neurog3* is re-expressed and *Neurog3*-derived cells give rise to islets, and (iii) silencing of *Neurog3* function prevents β-cell expansion (Xu et al., 2008).

The notion that β-cells double in mass after PDL, however, was recently put into question (Kopp et al., 2011). Total β-cell mass was previously determined using morphometric measurements (Solar et al., 2009; Xu et al., 2008). This calculation is

Table 7.1. Novel duct Cre-lines

Cre-lines	type of transgene	lineage tracing during[a] embryo-genesis	post-natal	PDL	neogenesis detected	possible problems	reference
CAIICreER	BAC with human promoter		x	x	yes	verification of faithful Cre expression still lacking	(Inada et al., 2008)
CK19^{CreERT}	knock-in, knock-out		x			low level labeling of islets	(Means et al., 2008)
Hnf1bCreER	BAC	x	x	x	no		(Solar et al., 2009)
Muc1^{IC2}	knock-in, IRES	x	x	x[b]	no	only ~10-15% duct labeling, and marks acinar cells	(Kopinke and Murtaugh, 2010)
Hes1^{C2}	knock-in, knock-out	x		x	no		(Kopinke et al., 2011)
Sox9CreER	BAC	x	x	x	no		(Kopp et al., 2011)
Sox9$^{IRES-CreERT2}$	knock-in, IRES	x	x	x	no	different lineage tracing results in acinar pancreas than Kopp et al	(Furuyama et al., 2011)

[a]x denotes whether Cre-lines were used for lineage tracing at the indicated time points and PDL. [b]Results of PDL lineage tracing using *Muc1^{IC2}* presented in Chapter 4.

based on determining total endocrine area divided by the total area (including islets, ducts and acini), measured over several representative sections, and multiplying by pancreatic weight. It does, however, not take into account the fact that the acinar tissue, which makes up the majority of pancreatic cells, is completely lost. Thus, the denominator for the ligated portion is automatically much smaller, resulting in an artificial increase of total endocrine area. Using a different approach, Kopp et al. measured whole pancreas insulin content using ELISA and showed that total β-cell mass did not increase after PDL (Kopp et al., 2011). Thus, it is questionable whether β-cell mass really increases after PDL and, if it does, whether it actually doubles.

It was also suggested that PDL-induced neogenesis was a result of *Neurog3*-expressing ducts turning into new β-cells and it was shown that siRNA knock down of *Neurog3* inhibited β-cell proliferation as well as their expansion (Xu et al., 2008). Since then, however, *Neurog3* has been found to be expressed by mature β-cells and actually to be required for their maturation and maintenance (Wang et al., 2009). Therefore, it is possible that the β-cell expansion defect observed after *Neurog3* knockdown is due to the functional role of Neurog3 in β-cells and not due to disrupted neogenesis.

Additionally, FACS analysis suggested that a 3-month-old pancreas contains ~500,000 β-cells (Dor et al., 2004). If we assume that ~200,000 β-cells are initially present in the ligated portion, this number should double to ~400,000 one week after PDL. During embryogenesis, one *Neurog3*-expressing cell normally gives rise to only one endocrine cell (Desgraz and Herrera, 2009). If we assume that this also applies to the PDL model, ~200,000 *Neurog3*$^{+}$ cells are therefore needed to achieve doubling of beta-cell mass. The original PDL study by Xu et al. suggested that around 5000 *Neurog3*-

expressing cells are present within one ligated pancreas at the 7 day time point, and of those 65% (3250 cells) were negative for endocrine markers (Xu et al., 2008). To identify *Neurog3*$^{+}$ cells, however, this study used a GFP reporter line instead of antibody staining. Since the GFP protein has a long half-life, >24hrs (Li et al., 1998; Xu et al., 2008), it is difficult to determine how many are actively expressing *Neurog3* at any given time point between the surgery and the analysis. Even if we assume that ~5000 *Neurog3*$^{+}$ cells are generated every day for 7 days (~40,000 cells), it is questionable whether this would be enough to cause doubling of β-cell mass.

Alternatively, neogenesis could indeed occur but at a frequency too low to be detected with any of the new Cre lines (Table 7.1). If we assume that ~40,000 *Neurog3*$^{+}$ ducts turned into new β-cells, we can make the following predictions of how many lineage-labeled islet cells we and others should have detected. With our *Muc1*^{IC2} (Chapter 2) and *Hes1*^{C2} (Chapters 3 and 4) lineages, between 10% and 20% of ducts were marked, respectively, so that around 4,000 to 8,000 *Neurog3*-derived β-cells should have carried the lineage label. Even more duct labeling is seen with *Hnf1bCreER* (~ 40%) (Solar et al., 2009) and *Sox9CreER* (~60%) (Kopp et al., 2011), which should have resulted in labeling of around 20,000 β-cells. Therefore, it seems unlikely that all Cre-lines combined missed a potential duct-to- β-cell differentiation event. The fact that *Neurog3* expression within *Sox9*- and *Muc1* lineage-derived ducts can be found but without detectable neogenesis (Chapter 2 and Kopp et al., 2011), rather suggests that *Neurog3*$^{+}$ ducts do not adopt a β-cell fate after injury.

The possibility of a "missed" duct cell, which might be special, however, is always given when using the imperfect TM-CreERT system (Kushner et al., 2010).

Additionally, it is formally possible that a yet-unidentified duct population might exist, which represents a source for new β-cells but does not express any of the above mentioned markers. Arguing against this hypothesis, several studies demonstrated that Sox9 (Kopp et al., 2011), Hnf1b (Solar et al., 2009) and Muc1 (Kopinke and Murtaugh, 2010) are expressed throughout the ductal epithelium. In addition, we stained ducts with different combinations of the above mentioned duct markers and found no difference between them. It therefore seems questionable that all five different Cre lines used to analyze PDL missed a unique duct cell type, responsible for neogenesis.

Based on our arguments, we suggest that PDL does not cause neogenesis and it is doubtful whether β-cell mass actually increases during PDL. One way of testing a potential increase in new β-cells after PDL would be to use ”pulse-chase” lineage tracing of pre-existing β-cells, which previously demonstrated that replication of these cells is the main mechanism for their postnatal expansion and maintenance (Brennand et al., 2007; Dor et al., 2004; Teta et al., 2007).

In the third part of this thesis, we assessed the differentiation potential of *Hes1*$^{+}$ cells in the embryonic and adult pancreas. For this, we made use of a new *Hes1*^{C2} knock-in allele that allows us to inducibly mark and trace *Hes1*$^{+}$ cells and their descendants. Our lineage tracing studies indicate that *Hes1* is indeed expressed in multipotent, Notch-responsive progenitors, of the early embryonic pancreas, which give rise to all three major cell types. At later embryonic stages, however, we find that *Hes1* marks bipotent, exocrine-restricted progenitors. In the adult pancreas, we confirmed earlier findings that Notch-*Hes1* signaling is active in a subset of duct and centroacinar cells (CACs). Through long-term lineage labeling, however, we could show that, similar to *Muc1*$^{+}$

cells, *Hes1*$^+$ duct cells do not generate β-cells after birth. This observation also held true after PDL, suggesting that *Hes1*$^+$ cells do not represent stem-like cells held in reserve for β-cell neogenesis during injury and regeneration (Chapter 5 and Fig. 7.1).

Finally, my finding that Notch promotes duct specification of exocrine-restricted progenitors in the embryo suggested that the differentiation status of adult *Hes1*$^+$ cells might also be controlled by Notch. By using a floxed deletion allele of *RbpJ,* which encodes the transcription factor partner through which Notch activates its target genes, we conditionally blocked Notch signaling in adult *Hes1*$^+$ cells. We found that acute loss of *RbpJ* in adult *Hes1*$^+$ CACs causes their rapid transdifferentiation into acinar cells. An acute pancreatitis injury model further indicates a physiological role for wild type CACs in assisting acinar cell regeneration (Chapter 6 and Fig. 7.1). This finding is the first evidence of an endogenous genetic program to control interconversion of cell fates in the adult pancreas.

Although classical studies suggested that islet cells arise from ducts in the embryonic pancreas (Pictet and Rutter, 1972), it remained unclear whether these duct-like structures are truly the precursors of the adult ducts. In addition, the contested question of whether adult duct cells ever give rise to islet cells could not be answered except through a genetic lineage-tracing approach. This thesis utilized two novel mouse lines that, for the first time, permitted definitive lineage-tracing of embryonic and adult ducts to prove the ductal origin of embryonic islet cells and to refute the possibility of exocrine-to-endocrine conversion in the adult pancreas. While converting the exocrine pancreas into new beta-cells could provide in principle an attractive method to treat human diabetes, the lack of an endogenous islet stem cell suggests that such an approach

will require genetic reprogramming of exocrine cells, either in vivo or in vitro (Cohen and Melton, 2011; Pan and Wright, 2011; Zhou et al., 2008).

This thesis also addressed the long-standing question whether the pancreas contains an adult stem/progenitor cell (Bonner-Weir and Weir, 2005). While centroacinar cells have been proposed as a potential progenitor cell population (Rovira et al., 2010; Yanger and Stanger, 2011), our novel Hes1-Cre line provided the first in vivo evidence that these do indeed represent a facultative progenitor population in the adult pancreas. We further showed that these cells utilize Notch signaling for maintenance of their undifferentiated state and that they contribute to new acinar cells following pancreatitis injury. Notch signaling is not only required for successful regeneration after pancreatitis (Siveke et al., 2008) but is also implicated in pancreatic cancer (De La O et al., 2008). We, therefore, hope that our work will not only help in the development of new cures for acute and chronic pancreatitis but also serve as a starting point to elucidate its possible function in cell fate determination and plasticity in pancreatic cancer.

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